

# Conference on Immunology of Carcinogenesis

**Monograph 35**





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# **Conference on Immunology of Carcinogenesis**

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FRANK J. RAUSCHER, JR., *Director, National Cancer Institute*

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# CONFERENCE ON IMMUNOLOGY OF CARCINOGENESIS

Conference held by the Oak Ridge National  
Laboratory at Gatlinburg,  
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## OPENING REMARKS

One of the pleasant duties of the Director of the Oak Ridge National Laboratory is to offer "Opening Remarks" at Gatlinburg Conferences. Many of you who have been at other Gatlinburg Conferences are aware of this function of mine.

At this time, it's a particular pleasure to offer these opening remarks, since the topic, "Immunology of Carcinogenesis," is one of those timely subjects that has great practical implications. Its importance has been recently recognized by the establishment of the national cancer campaign. We're sorry that Dr. Rauscher, the new head of the cancer campaign, is unable to be with us.

For some of you, the Oak Ridge National Laboratory is that institution which sponsors Gatlinburg meetings, and indeed this is one of our traditions. But in addition to sponsoring meetings, the Oak Ridge National Laboratory is a huge energy laboratory, with interests that go beyond nuclear energy; it is a very large environmental laboratory as well—which of course makes sense, since, as *The Environmental Handbook* says, "All power pollutes."

All of this bears on Oak Ridge's interest in cancer and in carcinogenesis, for our whole attitude toward low-level environmental insult, whether it be radiation or chemical, will surely be modified once cancer becomes a fully controllable disease.

I mention the analogy of how our attitude toward residual pathogens in the environment was modified by the introduction of antibiotics, which allowed us to control infectious disease. Sanitation and public health reduced exposure to pathogens, and antibiotics did the rest. In a similar way, one expects technology to reduce environmental insults, and we technologists can do this. One hopes that biomedical science will cope with the eventual residuum of environmentally induced disease, such as cancer.

Let me mention one other point—as long as I have a captive audience! I am a technologist who understands the idiom of biology imperfectly. Nevertheless, I'm struck by the extraordinary recent findings relative to defense mechanisms against environmentally induced disease.

Just as biomedical science underwent a revolution when Louis Pasteur discovered that pathogens caused disease, and a host of later workers discovered how, at the cellular level, the body can mobilize defenses against such injury, so we appear to be entering a corresponding phase with respect to environmentally induced disease. We are beginning to discover mechanisms at the cellular level that protect against such disease, just as surely as at the cellular level we have discovered, long since, mechanisms for protecting against infectious diseases.

For example, at one of these Gatlinburg Conferences not long ago, we heard about the beautiful DNA-repair mechanisms that work, at the molecular level, against both radiation and carcinogens: At the cellular level there seem to be these immunologic mechanisms which are, of course, the subject of this Conference.

But I stray from my opening remarks. My main purpose is to welcome you to this Conference on behalf of the Oak Ridge National Laboratory, and this I now do officially and warmly. I trust the Conference will be highly successful, as have the previous ones in this series, and that we shall see you in Tennessee again soon.

Alvin M. Weinberg





## PREFACE

For the second time in this century, oncologists are turning to immunology as a possible means of reducing the incidence and morbidity of human cancer. The discouraging results of the first attempt to achieve this goal led to the widespread belief, held through the 1930's and 1940's, that immunology had little to offer cancer research. This negative attitude about tumor immunology was so strong that Ludwik Gross's demonstration of the development of specific tumor immunity in mice against chemically induced syngeneic tumors<sup>1</sup> was ignored for almost a decade. The idea that host defenses could be mounted against neoplasms was not generally accepted until the 1960's, after the confirmation and extension of Gross's work by Foley<sup>2</sup> and Prehn and Main<sup>3</sup> and the demonstration that virus-induced tumors also contained antigens that provoked host responses against the tumor.<sup>4,5</sup> Fortunately, the organizers and participants of this Conference did not have to concern themselves with the question of whether or not tumor-specific antigens exist. Rather, they were invited to discuss possible means of using these antigens as a basis for studying the etiology, diagnosis, prevention, treatment, and prognosis of cancer. In fact, the reinforcement of these discussions was established by 3 challenges presented by Dr. F. J. Rauscher, Director of the National Cancer Institute. These challenges were: 1) What can immunology do for the prospective cancer patient from the point of view of detection and diagnosis? 2) What therapy can immunology provide for the patient with an established tumor or with widely disseminated tumor? 3) Against what type of tumors and under what conditions can immunotherapy be directly applied with demonstrable beneficial effects?

The need for reliable methods of determining the status of specific tumor immunity of cancer patients served as the impetus to organize the first session of the Conference. Although several methods for the detection of immune response to tumor are available, none of them yields unequivocal information about the ability of the host to survive.

The question of whether tumor-specific antigens can be detected in tissues undergoing carcinogenesis but which are not yet malignant, and the immunochemical properties of tumor-specific antigens, were evaluated in sessions 2 and 3. These points are relevant to diagnosis, prevention, and treatment of cancer and develop a bridge to the molecular biologic aspects of malignant cells. Within these topics of expressed specificities is the aspect of "retrogenic" expressions as suggested by similarities shared by fetal and neoplastic tissues.

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<sup>1</sup> GROSS L: Intradermal immunization of C3H mice against a sarcoma that originated in an animal of the same line. *Cancer Res* 3:326-333, 1943.

<sup>2</sup> FOLEY EJ: Antigenic properties of methylcholanthrene-induced tumors in mice of the strain of origin. *Cancer Res* 13:835-837, 1953.

<sup>3</sup> PREHN RT, MAIN JM: Immunity to methylcholanthrene-induced sarcomas. *J Natl Cancer Inst* 18:769-778, 1957.

<sup>4</sup> HABEL K: Resistance of polyoma virus immune animals to transplanted polyoma tumors. *Proc Soc Exp Biol Med* 106:722-725, 1961.

<sup>5</sup> SJÖGREN HO: Further studies on the induced resistance against is transplantation of polyoma tumors. *Virology* 15:214-219, 1961.

Consideration of whether the host immune response is an important factor in the carcinogenic process and how humoral and cellular factors of tumor immunity affect neoplastic growth formed the bases of sessions 4 and 5. The past literature on these topics allow the reasonable conclusion that components of the immune system effectively modify the course of tumor pathogenesis. However, the complex interrelationships and feedback mechanisms of the immune system are poorly understood and limit exploitation of the immune phenomena for prevention and effective therapy. The mission of session 6 was an evaluation of whether tumor cells or the immune response to them can be modified to benefit the host.

The Conference was consolidated in session 7 within which the participants addressed themselves to the question: Are tumor-specific or tumor-associated antigens of value in the diagnosis and prognosis of cancer? Immunologic studies have contributed to many aspects of cancer research, but at present only immunotherapy offers the possibility of reducing the morbidity of human cancer. It is relatively easy to prevent cancer in animals by immunizing them with appropriate vaccines before challenge. Clearly, prevention of human cancer would be preferable to therapy. Unfortunately, there are now no safe and effective anticancer vaccines for human use and their availability in the near future seems unlikely. Certain forms of effective cancer immunotherapy do not require a knowledge of cancer etiology or of the anti-cancer mechanism. The portion of tumor immunology dealing with therapy is ably represented in this Conference by both animal and clinical investigations. A major impetus to this session was the demonstrated successful immunotherapy of human skin cancers with long-term cures by methods which in animal models involve activation of the macrophage-histiocyte component of the immune system.

Although the answers to the questions posed by the organizers of this Conference are less than complete, the reader of this volume will be rewarded by obtaining a clear picture of what the problems of tumor immunity are, where the field is heading, and what can be done now to reduce the incidence and morbidity of human cancer.

The Conference was sponsored by the National Cancer Institute and organized by the Carcinogenesis Section of the Biology Division of Oak Ridge National Laboratory. The organizing committee is indebted to Mr. J. R. Gilbert for help in ensuring the smooth operation of the Conference and the rapid publication of the proceedings. Special acknowledgment is given to C. E. Normand of Personnel Services Department, Oak Ridge National Laboratory.

H. J. Rapp  
M. G. Hanna, Jr.

**SESSION 1**

**Methodology for Detection of Antigens in  
Preneoplastic and Neoplastic Tissues**

**Chairmen: Tibor Borsos and Herbert J. Rapp**





## **Introduction: Methodology for Detection of Antigens in Preneoplastic and Neoplastic Tissues<sup>1</sup>**

**Tibor Borsos, *Biology Branch, National Cancer Institute,<sup>2</sup> Bethesda, Maryland 20014***

First I would like to make one request of you. Dr. Weinberg alluded to low-level environmental insults, and those of you who smoke ought to recognize that you are doing that. So if possible, I would like to ask that all smokers sit in the back of the room. You don't know how insulting it is to those of us who don't smoke, when you smoke; and if you did not know it, now you do!

Session 1 concerns itself with techniques. I think it very appropriate that a meeting on Immunology of Carcinogenesis starts with a discussion of techniques to detect tumor antigen. Good techniques are so important that, I, as an old immunochemist, believe that, in research, technique is probably 80 %, sweat 19 %, and brain power 1 %.

In tumor immunology, by the definition of these words, we must deal with antigens of cancer cells and the response of the host to these antigens. It is now quite clear that virtually all tumors one studies contain some substances which become antigenic either by our intervention or by experiments of nature. These antigens can be detected either by the host responding *in vivo* or by our manipulation *in vitro* of their response. Hence the division of session 1 into *in vivo* and *in vitro* techniques of detection of tumor antigens.

There's a second division of this session: We shall talk about humoral response and cellular response to these antigens. I think these divisions are well taken, although probably somewhat artificial.

All these techniques that we will discuss are designed to detect antigens. They serve as a tool to determine the difference between a normal cell and a transformed cell—the antigenic result of malignant transformation.

It should be quite clear to all of us that, when we use these techniques, we are not addressing ourselves to the animal's ability to handle its own tumor load. I think it important that we should realize that experiments designed to detect the difference between normal cells and malignant cells may have little relevance to the question whether the elimination of an already existing tumor by immunologic means is possible. Even the prevention model is included in this category, for a prevention model requires the vaccination of animals with tumor antigens before the growth of a tumor. Because of this, prevention is not yet possible in humans. This brings me to the ultimate aim of our meeting—the successful immunotherapy in those who already

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<sup>1</sup> Presented at Conference on Immunology of Carcinogenesis, held by the Oak Ridge National Laboratory at Gatlinburg, Tenn., May 8–11, 1972.

<sup>2</sup> National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

have cancer. Since we cannot yet prevent the transformation of normal cells or eliminate environmental hazards, we are faced with the task of devising methods to help the patient to handle his cancer. That is our challenge, and that is why I'm talking about immunotherapy.

Unfortunately, to my knowledge—and I'm sure there will be some who disagree with this statement—none of the techniques and tests we are using at the moment (be they *in vivo* or *in vitro*, be they humoral or cellular type) detect the immune status of an animal with respect to its ability to handle its tumor load, except those tests designed specifically for immunotherapy. For example, they may give some information about whether the lymphocytes are able to recognize an antigen, they may give some information about the macrophage's ability to kill, and they may show enhancement of tumor growth by humoral antibodies. But the ultimate test of success in what we want to achieve (*i.e.*, to help the patient who already has a tumor) is a model in which one takes an animal that has a cancer and manipulates it in such a way that it can rid itself of the tumor. So the substance of my introduction is that we should always keep in mind that, whenever we use these sophisticated techniques, either outside the animal or inside the animal, if the animal does not have a tumor before our intervention, we do not really study a model for human cancer. That is my message.

## ***In Vitro* Methods for Assessment of Antibody-Mediated Tumor Immunity<sup>1, 2</sup>**

Nelson L. Levy, D. Bernard Amos, Gregory V. Solovieff, and Ataulpa P. dos Reis,<sup>3</sup> *Division of Immunology, Duke University, Durham, North Carolina 27710*

**SUMMARY**—The advantages and disadvantages of several *in vitro* assays for antitumor antibody were discussed. The procedures considered were immunofluorescence, complement fixation, mixed agglutination, radio-immunoassays, cytotoxicity, and precipitin tests. A new procedure to provide xenoantibody-free complementation of the cytotoxicity test was described.—*Natl Cancer Inst Monogr* 35: 5–11, 1972.

ANTI-TUMOR antibody and the possibility of immunotherapy with such antibody were the tumor immunologist's principal objects of study until cell-mediated immunity began to receive increasing attention. Although antitumor antibodies are believed to be less important in the defense against solid tumors, and indeed, through their blocking effects are being cast as villains, they still have considerable relevance to the study of tumor-associated and tumor-specific antigens. While this report will discuss some of the more widely used or more innovative procedures, it is necessary to introduce this report with a caution about the dangers of all serologic reactions, *i.e.*, the problems of specificity and cross-reactivity.

Typically, an antibody has its greatest binding affinity to its own specific antigen. It may also cross-react to varying degrees with other antigens. How-

ever, so great may be the binding affinities that it becomes extremely difficult to distinguish specific binding from cross-reactive binding. In our own studies with HL-A antigens, we found some antibodies had very extensive and complex cross-reactivities, while others had a more restricted range of reactions. The need to make quantitative distinctions between specific- and cross-reactivity in studies on tumor antigens has not been greatly stressed. Calorimetry, differential heat, or *pH* gradient elution offers the possibilities of making the necessary distinctions in the tumor field as well as in the definition of histocompatibility antigens.

A wide variety of serologic procedures has been adapted to study the antigens of tumors. Each technique has some technical or theoretical advantages and also certain limitations. The choice of any individual procedure depends on the nature of the individual antigen and antibody under study.

In their original studies, Gold and Freedman (1,2) used classical immunologic procedures to demonstrate, on the surface of human colonic tumor cells, antigens not found on autologous normal colon. Rabbit heteroantisera against the neoplastic tissue was absorbed with autologous colon. The antisera formed precipitin lines with extracts of the tumor tissue and, as has subsequently been shown, with extracts of all tumors of entodermally derived gut epithelium as well as of normal fetal gut tissues. Building on these studies, Krupey *et al.* (3, 4) partially characterized chemically the carcinoembryonic antigen (CEA) and developed sensitive

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<sup>1</sup> Presented at Conference on Immunology of Carcinogenesis, held by the Oak Ridge National Laboratory at Gatlinburg, Tenn., May 8–11, 1972.

<sup>2</sup> Supported by Public Health Service research grants CA13070 from the National Cancer Institute and AI08897 from the National Institute of Allergy and Infectious Disease. Also supported in part by Public Health Service research grant GM10356 from the National Institute of General Medical Sciences, and Institutional grants from the American Cancer Society, Inc. Nelson L. Levy is the recipient of Public Health Service Special Fellowship 1 F11 NS02320 from the National Institute of Neurological Diseases and Stroke.

<sup>3</sup> *Present address:* Universidade Federal de Minas Gerais, Belo Horizonte, Brazil.



radioimmunoassays (5) to detect circulating CEA in tumor patients. Studies on antigens similar to CEA are being conducted, and the complexity of the antigens, and their cross-reactivity with normal tissues, are becoming apparent. However, 2 factors have facilitated the studies on CEA: 1) the availability of large amounts of autologous normal tissue with which to absorb the heteroantisera, since generous normal tissue margins are routinely excised in colonic cancer surgery and 2) the nature of the CEA itself, *i.e.*, its strong antigenicity and the ease with which it is extracted and purified.

Such privilege has not been afforded workers studying the antigens of most other human tumors, and techniques for detecting weak tumor antigens on the intact cell and requiring only small amounts of serum antibody have been needed. Immunofluorescence (IF) has been used in many experimental and human tumor systems. Studies on Burkitt's lymphoma and related neoplasms by the Kleins, Henles, and their associates illustrate the extensive application of this technique (6-9). Cells fixed with acetone become permeable to fluorescein-labeled antibody and allow demonstration of internal antigens, such as the Epstein-Barr virus (EBV) capsid antigen and the EBV-associated early antigen (6, 7). Unfixed living Burkitt cells are used to identify the antigens associated with the plasma membrane (8, 9).

With direct IF, the test antibody itself is labeled with fluorescein or rhodamine and reacted with tumor cells on a coverslip. This procedure requires the isolation and labeling of each antibody under study and, for general screening procedures, has been supplanted by indirect IF. Here, the cells are reacted with unlabeled test sera, and cell-bound antibody is detected by a second reaction with fluorescein-labeled anti- $\gamma$ -globulin. Nonspecific fluorescence, however, is a major problem in the interpretation of any IF data. This has been largely overcome by use of the ability of unlabeled antibody to block competitively the direct IF reaction (9). IF is, then, a versatile tool of wide applicability. It requires no elaborate procedures for the preparation of antigen and very few cells—even tumor imprints being adequate for some studies. The major drawbacks are that IF is relatively insensitive, is plagued by problems of specificity, and, quite importantly, gives no information on the functional activity of the antibody detected.

Complement fixation (CF) has been used infre-

quently to study membrane-associated tumor antigens. This is largely due to the difficulty of preparing suitable test antigens lacking significant anti-complementary activity. The nature of the anti-complementary products of many subcellular preparations deserves further study. Eilber and Morton (10) detected antisarcoma antibody in the sera of 92% of sarcoma patients versus 6-20% in various control groups. They extracted the test antigen from disintegrated tissue-cultured sarcoma cells. Their studies required the use of human cord complement and illustrate another problem with CF, that of finding an effective complement source. Chan *et al.* (11) also found a requirement for human complement in the reaction of serum from breast cancer patients with antigen from a lymphoid cell line.

The tumor immunologist must cautiously interpret CF data. Since the antigen extraction procedures do not allow differentiation between antigens derived from the cell surface and those from the intracellular compartment, data on CF reactivity may not correlate with transplantation studies. Thus CF studies showing antitumor reactivity should be confirmed by subsequent removal of this reactivity from the test serum by absorption with intact cells.

A useful technique in the study of several tumor systems has been mixed agglutination (12), in which sensitized red blood cells are attached to specifically sensitized tissue culture cells by antiglobulin. It offers increased sensitivity over the above techniques. Although costly in its requirement for tissue-cultured cells, it is suitable for screening large numbers of sera. Since intact cells are used, one can be confident of studying only surface antigens. Quantitation of results is easier than with fluorescence and requires no elaborate facilities. Like fluorescence, however, this technique can present specificity problems and does not provide information on the functional nature of the antibody under study.

Cytotoxicity assays, in which damage to sensitized cells by complement is measured by direct cell counting, dye uptake, or release of fluorescein or of a radioactive isotope such as  $^{51}\text{Cr}$ , are widely used to detect antibody to histocompatibility antigens (13-15). They offer 2 major advantages to the tumor immunologist: 1) They detect only surface antigenic components and should, therefore, correlate with transplantation data, and 2) they can

provide information on the functional nature of the antibody detected (16). The latter point gains added significance as we attempt to dissect the antibody activity of the tumor patient, not into the routine immunoglobulin classes and subclasses, but into blocking and unblocking factors and into cytotoxic, cytophilic, and lymphocyte-associated antibodies.

Cytotoxicity assays are affected by several problems, however. They are relatively insensitive and may fail to detect activity discernible by such assays as mixed agglutination or fluorescence (17). The effectiveness of the assays is largely a function of the efficiency and specificity of activation of the complement system. The work of Baker *et al.* (18) suggested that, as in the red blood cell system (19), fixation of C1 by nucleated cell-antibody complexes requires only one IgM molecule but two proximate IgG molecules. Moreover, their studies (18) showed that, while only one cytotoxic site is necessary to lyse a nucleated cell, only one such site develops per thousand or more C1 fixation sites. Thus, a cell may have many bound antibody molecules but fail to be lysed in a cytotoxicity assay. Considerable difficulties also arise from the complement source itself. Two major sources of difficulty can be identified: 1) Complement from certain species cannot induce lysis, and 2) natural antibodies in the source of complement frequently interfere with the test. Fresh serum from the guinea pig or man, ordinarily a source of abundant hemolytic complement activity, may be ineffective in the lysis of nucleated cells. This is especially true of human systems. Rabbit serum, while relatively impotent in a hemolytic system, has been the most consistently effective source of complement lytic to nucleated human and mouse cells. However, it contains potent natural xenoantibodies to the cells of many species, including man (20). These xenoantibodies may, with many tumor cell targets, themselves produce cytotoxicity and thus reduce the specificity of the assay system.

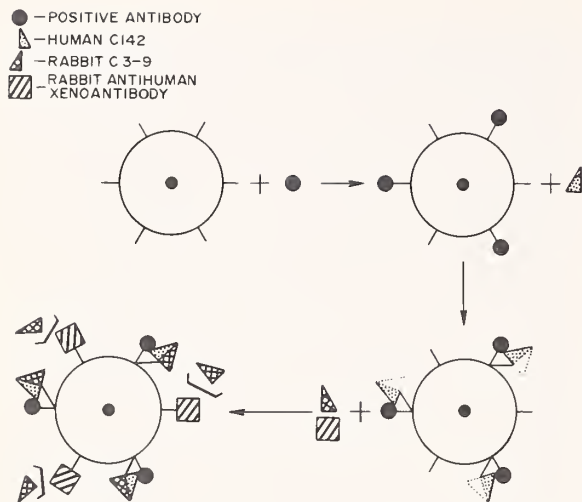
While the recent studies of Ferrone *et al.* (21) suggested that it may indeed be the synergistic action of the rabbit xenoantibody with the sensitizing antibody that renders rabbit complement so effective in nucleated cell lysis, most workers using the cytotoxicity assay have tried some means to rid the rabbit serum of this xenoantibody activity. Laboratories using cytotoxicity to detect histocompatibility antigens have little obvious problem with the xeno-

antibody, since normal lymphocytes are relatively insensitive to the xenoantibody (22). Detection of antigens on tumor cells, leukemic cells, and stable tissue culture cell lines has imposed more rigorous demands, however. Simple dilution may reduce the concentration of xenoantibody until it is no longer lytic but frequently renders the complement itself ineffective (14). Column chromatography to remove the xenoantibodies could be considered, but coincident removal of various complement components complicates such attempts. Absorption of the rabbit serum with red blood cells is ineffective (20). Absorption with the specific target cells to be used in the cytotoxicity assay (20) or with agar (23) may remove the xenoantibody activity, but only with great difficulty, and may require very large numbers of cells. There may even be some degree of specificity in the reactions of xenoantibodies, and absorption may need to be repeated for each type of target cell used. Depletion of complement activity during absorption can also be a problem but may be minimized if the absorptions are performed in the presence of ethylenediamine-tetraacetate, as suggested by Boyse *et al.* (24).

Herberman (25) used a somewhat different approach by adding to whole rabbit serum a small amount of purified human IgM. This procedure yielded a complement source apparently free of natural cytotoxins when tested on several lines of normal and neoplastic lymphoid cells. However, it has not been as effective in removing cytotoxins to various solid human tumors (R. Herberman and P. Wright, personal communication).

We have been particularly interested in comparing the immunochemical nature of blocking and cytotoxic antibodies to human brain tumors. The problems discussed above have made difficult our titration of cytotoxic antibody. Since these problems are likely to be encountered by other investigators working with cytotoxic antibodies to human tumors, we shall fully discuss our solution to these problems. Rather than attempting to remove or inactivate the xenoantibody, we tried to circumvent its biological effect. Human complement is active in a hemolytic system, and its ineffectiveness, relative to rabbit serum, in a nucleated cell lytic system cannot be explained by a paucity of any of the individual complement components (26). We therefore speculated that the potency of rabbit serum lies in a substrate difference at the terminal lytic component level, wherein guinea





TEXT-FIGURE 1.—Conceptual representation of a xenoantibody-free complementation system for the cytotoxicity assay. Sensitized human cells were incubated with human initial complement components (C142). A complex of activated C4 and C2 formed the enzyme C3-convertase which was able to cleave C3 and thus lead to terminal component activation. Since the human components were homologous to the target cells, they contained no natural antitarget cell antibody. The cells were then incubated with whole rabbit serum treated to inactivate the initial components. The xenoantibody did get on the target cell but, without initial components, could not itself fix complement. Those sites where specific sensitizing antibody had been bound, however, did already have activated human C142 sites and thus could activate the terminal rabbit components and induce cytolysis.

pig, human, etc., enzymes are most effective against red blood cell membranes, whereas rabbit enzymes favor a substrate present on nucleated cell membranes. We therefore sought a method to activate only the terminal rabbit components so that, while the rabbit xenoantibody was allowed to bind to the target cells, it was prevented from itself activating the cytolytic complement cascade.

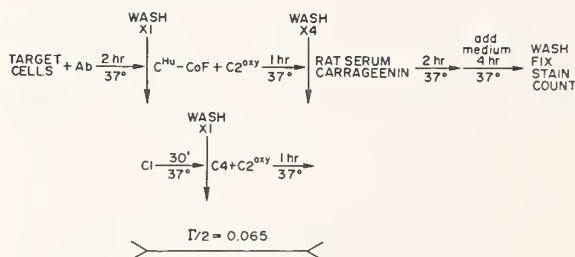
Text-figure 1 illustrates the principle of our approach and text-figure 2 the experimental details.

Text-figures 3, 4, and 5 show the effectiveness of this complementation system against normal human fibroblasts sensitized with monospecific anti-HL-A isoantisera. Whole rat serum produced lysis of 60% of the target cells, but the background of 25% in control tubes was unacceptably high (text-fig. 3). In experiments with whole rabbit serum, the background was even higher. With the system described in text-figures 1 and 2, with rat or rabbit

terminal components (text-figs. 3–5), no lysis was observed with control antibody. Moreover, neither the human first reagent nor the rat or rabbit second reagent alone supported lysis of sensitized or unsensitized cells.

Recent reports (29) showed that the terminal components may be activated through a third alternative pathway, independent of both C3-convertase and C3-proactivator. It is unlikely, however, that the xenoantibody, through this pathway, produced significant lysis, since at no time did cells not specifically sensitized exhibit lysis in our system. It is of interest that substitution of our 2-reagent complementation for conventional whole serum complement not only eliminated the background lysis but also augmented the lysis of specifically sensitized cells (text-figs. 3–5).

The sensitivity of the cytotoxicity test may also be increased by the reaction of the antibody-coated target cell with antihuman  $\gamma$ -globulin before the addition of complement. Such procedures have

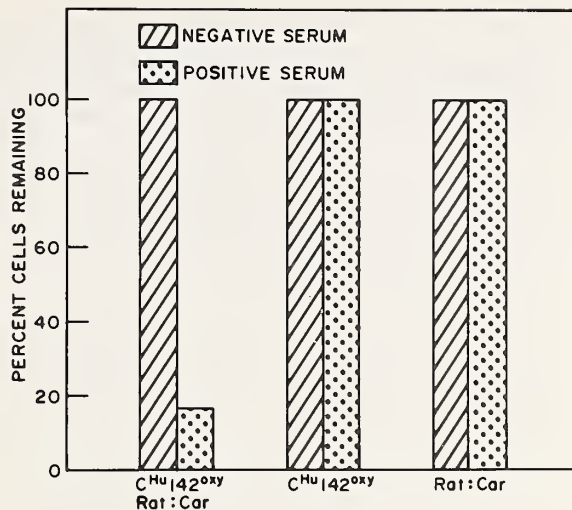


TEXT-FIGURE 2.—Procedure for xenoantibody-free complementation of the cytotoxicity assay. One hundred tissue-cultured target cells were seeded into each well of a Falcon Microtest II plate and allowed to adhere overnight. The cells were incubated with test antisera for 2 hours at 37°C and washed. Cobra factor-treated (26) whole human serum ( $C^{Hu}$ -CoF), supplemented with 60  $CH_{50}$  units of oxidized, purified C2, was added, and, after 1 hour at 37°C, the cells were washed 4 times with  $Ca^{++}$ - and  $Mg^{++}$ -free Veronal buffer. [ $C^{Hu}$ -CoF was supplemented with oxidized C2 (27) to prolong the half-life of the C3-convertase site.] Alternatively, sensitized cells could be incubated with 125  $CH_{50}$  units of purified C1, washed, and reincubated with a mixture of 250 units C4 and 60 units oxidized C2. During either initial component incubation an ionic strength of 0.065 was carefully maintained. Whole rat (or rabbit) serum containing 1000  $\mu g/ml$  of carrageenin (28) was then added, and the cells were incubated for 2 hours, after which, without removal of the serum, growth medium was added and the incubation continued for 4 more hours. The cells were washed, fixed with methanol, and stained with Giemsa. The total number of cells in quadruplicate wells was counted.

been used to increase the sensitivity of anti-HL-A sera (A. P. Reis and D. B. Amos, unpublished results). They do, however, negate one major advantage of the cytotoxicity assay—the ability to distinguish cytotoxic and noncytotoxic antitumor antibody.

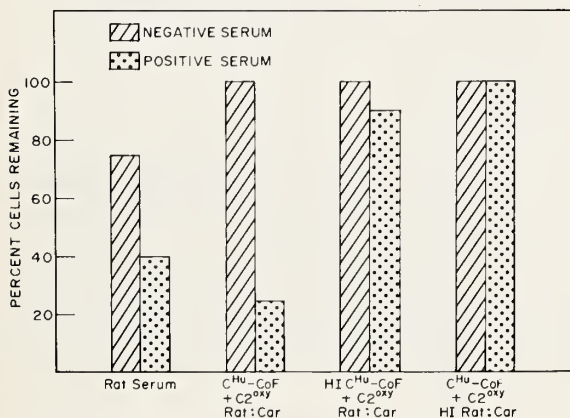
Enzymatic treatment of the target cells also may augment the sensitivity of any of the assay procedures, presumably by unmasking cryptic antigenic sites. Trypsin and neuraminidase (30) have been most widely used in this regard.

Unrelated to the type assay performed, a factor which can strongly influence the results is the variation in surface antigen expression with the mitotic phase of the target cell. Shipley (31) reported



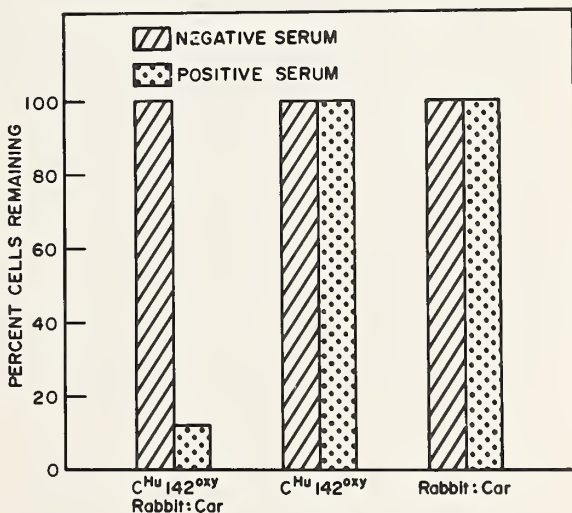
TEXT-FIGURE 4.—Lysis of sensitized fibroblasts with purified human initial complement components and carrageenin-treated rat serum. Same as text-figure 3, except that purified human C1, 4, and 2 were used in place of cobra factor-treated human serum.

The study of antitumor antibodies needs development along two lines. The importance of reliable, sensitive methods for the screening of large numbers of patient sera has been emphasized in the work of Thompson and his colleagues. Using a sensitive radioimmunoassay (5) they have been able



TEXT-FIGURE 3.—Lysis of sensitized fibroblasts with cobra factor-treated human serum and carrageenin-treated rat serum. Fibroblasts from an HL-A 9, 7, 3, Ao41 patient were sensitized with monospecific anti-HL-A 3 or control serum. Complementation was with either whole rat serum or the system described in text-figure 2. Additional controls received a heat-inactivated initial or terminal complement reagent.

that Chinese hamster cells, synchronized in culture by hydroxyurea, are most susceptible to immune cytolysis when in mid S phase. Consistent with this is the report of Chan *et al.* (11) that a lymphoid cell line (Belev) derived from a breast cancer patient carried antigens specifically reactive with the sera of several other breast cancer patients. Belev cells in stationary growth had 16 times as much extractable antigen as cells in the log phase. If this interesting finding is confirmed, negative results obtained with cultured cells, or with antigens extracted from such cells, must be re-examined with this information in mind.



TEXT-FIGURE 5.—Lysis of sensitized fibroblasts with purified human initial complement components and carrageenin-treated rabbit serum. Same as text-figure 4, except that carrageenin-treated rabbit serum supplied the terminal components.

to offer diagnostic and prognostic data on enterodermally derived adenocarcinomas; these data were based on the detection of circulating CEA. Clearly, radiolabeling techniques offer the best avenue to improving the sensitivity of tests for anti-tumor antibody. The use of radiolabeled C1 might improve the detection of complement-fixing antibodies, particularly in view of the  $>1,000:1$  ratio of C1 fixation sites to cytotoxic sites on nucleated cells (19). Similar arguments might apply to the use of radiolabeled C3. Ideally, however, one would like purified radiolabeled tumor-antigen preparations with which to perform assays analogous to the Farr technique (32). Experience with the Farr technique has emphasized the great sensitivity of such procedures, and the introduction of technical aids such as zirconyl phosphate gel (33), which binds  $\gamma$ -globulin and thus any associated labeled antigen, has made more feasible the application of these procedures to large-scale screening of sera. Considerable progress has been made in the purification and solubilization of histocompatibility antigens (34), and application of these techniques to tumor antigens is now in its early stages.

The other area needing further attention is the quantitative immunochemical analysis of tumor antigen distribution on the cell surface and of the binding properties of the various types of antitumor antibodies. The work of Boone *et al.* (35) with HeLa cells and radiolabeled anti-HeLa serum provides an excellent prototype for such studies. The use of Fab fragments rather than whole sera, proof that the reactions had gone to equilibrium, and double-labeling techniques, such as those employed by Day and Lassiter (36) to quantitate nonspecific protein binding, would improve such studies and should be incorporated in future analyses of tumor systems. Of particular importance would be such studies on the same tumor cells with both blocking and cytotoxic antisera. This might provide information on whether separate antigens are involved with each antibody type and, indeed, on the differences, if any, between the antibody moieties themselves. Recent work from our laboratory has suggested that the tumor-specific antigenic determinants involved in blocking differ from those involved in cell-mediated cytotoxicity. Immunochemical confirmation of this concept, would greatly encourage those interested in preparing tumor antigens capable of inducing nonblocking immunity to various

tumors and thus suitable for general immunoprophylaxis of cancer.

Finally, a word about the possible significance of anti-idiotypic antibodies in tumor immunology. Lewis *et al.* (37) described, in patients with advanced melanoma, antibody capable of binding specifically to auto-antibody against their own tumors. The biological significance of the former antibody is unknown. Possibly analogous to these findings is the presence of unblocking factors in the sera of some tumor patients who clinically are handling their tumors well (38). These factors, when mixed with blocking sera, abolish the latter's activity. The possibility is interesting that both unblocking factor and the antibody described by Lewis *et al.*, though perhaps of opposite biological significance, have anti-idiotypic specificities.

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## **In Vivo Methods for the Assessment of Antibody-Mediated Tumor Immunity<sup>1, 2</sup>**

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**SUMMARY**—Antibody-mediated tumor immunity can be assessed *in vivo* through the use of neutralization tests or by the technique of passive immunity. In neutralization tests, tumor cells are brought into close contact with antibody molecules before tumor implantation. This arrangement provides for high sensitivity but leaves open the question of the accessibility of tumor cell antigens to circulating antibody. Passive immunity is a less sensitive technique but it simulates more closely the conditions in which antibody and cell-surface antigens interact in established tumors. In both procedures, antibody is present only transiently and the rates of activation and mobilization of substances that mediate immune damage become limiting factors in determining the extent of tumor destruction by antibody. There is good evidence that complement-dependent cytotoxicity as observed *in vitro* is not important *in vivo* in mice, although several components of complement are essential for antibody-mediated immunity. Antisera specifically reactive with tumor cell antigens not only activate mediators of immune damage but also suppress the development of host responses to the antigens and perhaps also to interference with the action of host-derived immune substances on the tumor. Both these complex processes, immune destruction and enhancement, may be touched off by antibodies of the same class and specificity. Whether enhancement or immunity assumes greater importance in any situation depends on a number of variables, many of which are poorly understood.—*Natl Cancer Inst Monogr* 35: 13–18, 1972.

NUMEROUS procedures have been devised for the detection *in vitro* of antibodies or putative antibodies reactive with tumor-specific or tumor-distinctive antigens (1). These procedures have been of singular importance in the demonstration of the existence of unique or distinctive antigens in a variety of tumors that occur in man or in experimental animals, and they are of potential value in the diagnosis of some forms of cancer. They are

likely, moreover, to become useful as guides to new therapeutic regimens and to assume an important role in the prognosis of neoplastic diseases.

The multitude of tests described in recent years provides techniques of great diversity and of widely ranging applicability, and the trend to the use of microtechniques provides tests of remarkable sensitivity. So versatile have these tests become that it is now considered possible to study the phenomenon of immunologic enhancement *in vitro* (2).

These *in vitro* methods are thus impressive on many counts, and they will continue to be of great value in clinical investigations and in basic studies in the field of tumor immunology. Methods for the

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detection and measurement of antibody-mediated tumor immunity *in vivo* are, by comparison, time consuming and of limited applicability. It is, for example, often difficult to find susceptible hosts for primary isolates of tumors in species where inbred animals are not available and, for human tumors, *in vivo* methods can be employed only through the use of xenografts. Clearly, the continued use of *in vivo* techniques for the study of humoral antibodies in tumor immunity turns largely on the ability of these techniques to provide information of a kind not available from tests conducted *in vitro*. The obvious shortcoming of the latter lies in the difficulty of relating results obtained from them to the actual role of antibodies in tumor-bearing animals. This difficulty stems from the great complexity of systems which mediate immune damage to tissues *in vivo*. Tests carried out *in vitro* pay scant attention to mediators other than complement and even that system is represented under nonphysiologic conditions, especially insofar as it is obtained from species other than that from which the tumor and antiserum were derived. The enormously complex and interacting systems involved in immune damage to tissues *in vivo* are not likely to be duplicated *in vitro*, and the biological significance of antibodies specifically reactive with tumor cell antigens must, perforce, be assessed by studies with tumors growing in appropriate living hosts.

Unfortunately, there has been little scope for the development of new methods for assessing the role of humoral antibodies *in vivo* and there are still only 2 general and straightforward procedures available. Antiserum can be mixed with tumor cells *in vitro* and the mixture then be injected into susceptible hosts, or antiserum and tumor cells can be injected into the same animal at separate sites and at different times. The former technique is referred to as a neutralization test and the latter as passive immunity. Both methods date from very early studies and are among the simplest of all immunologic procedures to perform. The outcome of either type of test may be gauged in terms of the numbers or proportions of recipients which survive the injection of tumor cells or by periodic measurements of the growing tumors. The latter will obviously provide a more sensitive test for antibody, and for many weak tumor-specific transplantation antigens (TSTA) it may be the only means of detecting immunity. In some earlier studies on the effects of humoral antibody *in vivo*, target tissues,

including tumors, were placed in Millipore chambers that were inserted into the peritoneal cavities of serum-treated animals. The effects of antibody were determined by macroscopic or microscopic examination of the contents of the chambers at some arbitrary time after administration of the serum. The test is cumbersome, difficult to interpret and only rarely used at the present time. No further comment need be made on the purely technical aspects of these procedures and the principal line of discussion to be pursued here relates to the kinds of data which can be obtained through the use of the methods, the interpretation of such data, and their relationship to data obtained by *in vitro* methods.

A few studies have been reported in which it could be reasonably certain that the sera employed did contain antibodies reactive with TSTA and that such antibodies had a detectable effect on the outcome of tumor transplantation (3-5). However, the potential value of these techniques in the assessment of humoral antibodies in tumor immunity is more accurately gauged from the results of studies in which alloimmune or heterologous immune sera were employed. Since there is no difference in principle between tests carried out with antibodies specific for normal transplantation antigens and those carried out with antibodies specific for TSTA, the following discussion deals largely with tests of the former type.

The neutralization technique has the advantage of bringing small numbers of cells into close contact with relatively large amounts of antiserum prior to implantation of tumor. Thus, it can provide information on the sensitivity *in vivo* of cells that have combined with antibodies in circumstances in which diffusibility or concentration of the latter are not limiting factors. While this can establish in an absolute way the sensitivity of sensitized cells to humoral antibodies *in vivo*, it may in fact bring cells into contact with the latter at concentrations that are rarely, if ever, realized in the usual host-tumor relationship. Furthermore, since this procedure involves the action of antibodies on cells that have yet to acquire a vascular bed or other stromal elements, it leaves open the question of the accessibility of antibody and auxiliary immune substances to cells in established tumors.

The technique of passive immunity is generally less sensitive than the neutralization test, but it can be carried out so as to approximate more closely the conditions which obtain in actively responding



hosts. For example, when antibody is administered intraperitoneally or intravenously and tumor cells are implanted subcutaneously, the antibody must leave the vascular system in order to reach the target cells. If administration of serum is delayed until the tumor has acquired its own blood supply, then the conditions of the test are similar to those in which the cells of established tumors react with antibodies that have been generated by their hosts.

In short, neutralization tests are extremely sensitive but do not simulate closely the conditions in which tumor cells interact with specific antibodies in autochthonous hosts. Passive immunity can meet this last requirement to a large extent, but it is a less sensitive technique. Both methods can detect TSTA and can establish the sensitivity of cells to humoral antibody *in vivo*. There are, however, difficulties involved in the interpretation of data that are obtained through the use of these techniques.

In both neutralization and passive immunity tests, antibody is usually transferred at a single point in time, either in solution or in combination with cell-surface antigens. There is, accordingly, a limited period of time during which mediators of immune damage can act on the tumor cells. In the first place, the implanted cells are destined to undergo mitosis at a relatively high rate resulting in a dilution of the antibody at cell surfaces; in the second place, the transferred antibody undergoes metabolic decay so that decreasing amounts are available to react with antigens at the surfaces of new cells. The efficiency with which sensitized tumor cells are destroyed *in vivo* depends, therefore, on the rapidity with which mediators reach the site of implantation. Since complement is an essential primary mediator in many, if not all, forms of graft damage caused by humoral antibody, the concentration of appropriate components of this system and the ease with which the components are transported to the site of implantation are crucial in determining the outcome of these tests. That the concentration of some mediator, probably complement, can be a limiting factor in the destruction of sensitized tumor cells *in vivo* has been suggested in previous work in which it was shown that the number of cells used in neutralization tests was critical in determining the outcome of the tests, even though all of the injected material had come from a common pool of sensitized cells (6). Evidently, complement did not reach the implanted

cells in sufficient concentrations to cause damage to a large number of cells prior to the inception of cell division and only with relatively low doses of cells could neutralization be detected.

Recently, there has come to light an additional factor that may limit the efficacy of antibody that is present only transiently in tumor-bearing animals. Amos *et al.* described the gradual loss of sensitivity to complement in cells mixed with antisera and allowed to stand *in vitro* in culture medium (7). Other studies suggest that this may be due to the mobility of cell-surface antigens, a property which allows them to form immune aggregates which are subject to endocytosis (8, 9). Regardless of the mechanism of the phenomenon, it will, if it occurs *in vivo*, have considerable influence on the outcome of neutralization and passive immunity tests. Even in actively immunized hosts the rate of production of antibody and the ease with which it is brought to the sites of tumor cells may be insufficient to cause significant damage because of the limitations imposed by the availability of mediators and the loss of sensitization due to endocytosis or inactivation of antibody by other means. These limiting factors may also explain the frequent observation that vascularized tumors even though of small size are extremely difficult to destroy even when very large amounts of antibody are infused.

Mention is made at several points above of factors which mediate the destruction of target cells following their interaction with specific antibody. The precise nature of the mediator systems involved in tissue destruction of the type considered here is unknown, though a good deal of evidence indicates that the plasma-complement system plays a central role. In the destruction of sensitized tumor cells *in vitro*, this is indispensable, and an excellent correlation exists between the degree of sensitivity to complement *in vitro* and the ease with which cells can be destroyed by humoral antibody *in vivo*. It seems reasonable, therefore, to conclude that the latter process involves the participation of complement. Still, some caution should be exercised in interpreting the results of studies carried out *in vivo* strictly in terms of the mechanisms of cell damage that have been identified *in vitro*.

In the first place, studies conducted *in vitro* often make use of complement obtained from a species different from that of the origin of cells and antiserum. This not only leads to abnormal concentra-

tions of various components of the system but it may introduce errors of a qualitative nature; *e.g.*, it has been shown that mouse complement is not activated by IgM molecules but requires instead certain classes of IgG antibody. This is in striking contrast to guinea pig complement which is often used *in vitro* with mouse cells and alloantibody.

Secondly, a number of studies suggest that the cytotoxic properties of complement, as expressed in serologic tests, are of less importance in mediating immune damage *in vivo* than are other biologic properties of complement. We have recently studied the effects of mouse anti-rat sera on rat skin that had been grafted to mice whose immune responses had been suppressed by thymectomy and treatment with rabbit anti-mouse lymphocyte serum (10). When these xenografts were healed in and were expected to survive for 2 or more additional weeks, the antisera were injected intraperitoneally so that we were in effect studying the role of humoral antibody in tissue damage by the technique of passive immunity. As early as 15 minutes after the serum was injected, signs of inflammation were easily recognized in the grafts; within 2–4 hours, there was evidence of vascular breakdown with marked interstitial hemorrhage; by 24–36 hours most of the grafts were completely destroyed. This dramatic and highly reproducible sequence of events is completely inhibited if the graft recipients are selectively depleted of polymorphonuclear (PMN) leukocytes just prior to the injection of anti-rat serum. Thus in the presence of a functioning complement system the antibodies, though present in amounts 20-fold greater than needed to destroy grafts in control animals, were totally innocuous in the absence of PMN leukocytes. Cell damage cannot in this system be attributed to direct cytotoxic action of complement. That some components of complement (C) play an essential role in this form of antibody-mediated damage, however, is indicated by the observation that the injection of purified CoF from cobra venom can also inhibit the reaction. This substance activates and depletes C3 and reduces the concentration of other late-acting components; it has no detectable effect on PMN leukocytes, mononuclear cells, platelets, or clotting factors.

Further evidence for the participation of complement components comes from experiments carried out with graft recipients that have genetically determined deficiencies of C5. Their grafts can be

destroyed following the injection of antiserum but the process develops much more slowly than that observed in C5(+) mice. Total destruction of the grafts takes about twice as long to occur and in a significant percentage of cases the grafts become the sites of intense inflammatory responses from which they recover completely. In the latter cases the grafts are actively rejected by their hosts several weeks later when the hosts are recovering from the effects of the immunosuppressive treatment. On the basis of all of the information obtained in the study of this system it is concluded that C3 and PMN are essential for the production of tissue damage, and that C5 and later acting components of complement, though not indispensable, can accelerate and intensify the reactions leading to damage. The most likely function of complement in this process is to generate chemotactic substances that bring PMN into the graft. The granulocytes are likely to release substances directly responsible for tissue damage—just as they do in other forms of immune tissue destruction (11).

With respect to the relationship between the results of tests carried out *in vitro* and *in vivo* it is interesting to note that all our serologic studies with rat cells and mouse anti-rat sera pointed to the importance of all known components of complement in mediating the cytotoxicity initiated by humoral antibodies *in vitro*. *In vivo* this direct cytotoxic action of complement is not only non-essential but there is no evidence for its occurrence. An even greater discrepancy between data obtained in studies carried out *in vitro* and *in vivo* is indicated below in the discussion on enhancement.

We are currently studying the role of complement and other mediators in the destruction of allografts of tumors by humoral antibodies. We have been able to show that C5 is not essential for the neutralization of tumor cells, though the participation of other components is indicated by the results of experiments in which agents capable of depleting them *in vivo* have been employed. Additional work is obviously required for the elucidation of the precise mechanisms involved in the neutralization *in vivo* of sensitized tumor cells.

That C5 is not essential to the production of immune damage *in vivo* is also suggested by the observation that the immunosuppressive properties of rabbit anti-mouse lymphocyte sera are expressed with equal intensity in C5(–) and C5(+) mice. The depletion of thymus-dependent lymphocytes



does not, apparently, depend on direct cytotoxicity of complement (12).

The discussion up to this point has focused on the potentially destructive effects of antibodies because these are the properties most appropriately considered in relation to tumor immunity. It is well known, however, that humoral antibodies specific for antigens present in grafted tissues but absent in their hosts may not only fail to cause the destruction of the grafts but will, in fact, interfere with the development and expression of host responses which could effectively lead to rejection of such grafts. This phenomenon has been termed "immunologic enhancement" and it has been discussed in detail in a number of recent articles. No effort will be made here to review or restate these discussions; nor will there be any attempt to develop hypothetical explanations for the phenomenon of enhancement. I should like only to comment on some aspects of the problem bearing on the interpretation of tests designed to assess antibody-mediated tumor immunity *in vivo*.

Immunity and enhancement should not be viewed as phenomena that are necessarily mediated by antibodies of different classes or different specificities. Indeed, the evidence for the participation of antibody in the mediation of enhancement of tumor cells often comes from serologic tests which show that the antibody has complement-dependent cytotoxic properties. Furthermore, several antisera have been shown to cause destruction of one type of normal or neoplastic cell *in vivo* and to lead to enhancement of other types of cells of similar or identical genotype (13). There are even situations in which passive immunity may lead to apparent destruction of a fraction of transplanted tumor cells, which is followed by enhanced growth of the surviving cells (14). These observations are perhaps best explained by considering that the administration of antibody specific for graft antigens always leads to some degree of specific suppression of host responsiveness. This suppression then paves the way for prolonged or even progressive growth of tumor grafts. Suppression may or may not be accompanied by destruction of the graft, but, if it is, then enhancement will obviously not be observed. The degree of immunosuppression observed in a particular experimental situation will depend on a number of complex and interacting factors: the amounts, specificity, avidity, diffusibility, and biologic half-life of the antibodies; the nature and

concentration of the antigens in question; the site of the graft; and poorly understood aspects of host responsiveness. The extent of graft destruction attributable to passively acquired antibody will depend on the kinds and amounts of antibody, the concentration of cell-surface antigens, and the efficiency with which mediator systems are activated and mobilized. It is not surprising, therefore, that manipulation of the size of tumor cell inocula and of the amounts of serum injected can result in enhancement or immunity in a single experimental system.

Again, it may be noted that the results of *in vitro* tests are often poor indicators of the results of *in vivo* tests. It has been shown in several systems that tumor cells which are readily damaged by antibody and complement *in vitro*, may show enhanced growth in allogeneic recipients treated with preparations of the same antibody (3, 15). On the other hand it is entirely possible that a test carried out *in vitro* may fail to reveal the potentially destructive effects of some antibodies simply because the appropriate mediators are not provided in the *in vitro* system.

It is not being argued here that different classes of immunoglobulins may not be more or less efficient in mediating enhancement or immunity on the basis of the distinctive structural characteristics of the constant portions of their heavy polypeptide chains. These structural differences confer biologic properties which should influence the occurrence of the phenomena, but not all of these properties are so distinctive and contrasting as to permit categorical statements about the roles of various classes of antibodies in the complex phenomena of enhancement and immunity.

The injection of graft recipients with antisera specifically reactive with antigens, present in the graft but absent in the tissues of the recipient, can touch off 2 separate and complex series of events.

1) The reaction of antibody with cell-surface antigens activates primary mediator systems, principally complement. Depending on the concentration of antigens and antibodies at the cell surface and the chemical and biologic properties of the antibody molecules, various mediator systems are secondarily activated by altered components of complement. These systems interact in complicated ways that are not well understood and that generate toxic or potentially toxic substances.

2) The injection of antibody leads to suppression



of the development of host responses to graft antigens and perhaps also interferes with the action of host-derived immune substances on cells of the graft. The extent of suppression and interference is again determined by the kinds and amounts of antibodies injected and by the concentration of relevant antigens.

Obviously, slight alterations in experimentally controlled conditions or in a variety of factors, which are now beyond the control of the experimenter, can decisively influence the outcome of grafts placed in recipients receiving infusions of antisera. It is a matter of great practical importance and of considerable conceptual interest that we identify these variables and bring them under closer control.

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## DISCUSSION

**J. D. Feldman:** Dr. Winn, can you get any results, such as you noted, with an allogeneic antibody in an allogeneic system, that is, a tumor graft being rejected by an allogeneic antibody?

**H. J. Winn:** There is certainly a great deal of evidence that lymphomas, for example, can be destroyed by an allogeneic antibody. There is also evidence that some of the nonlymphomatous tumors, sarcomas and carcinomas, can be destroyed, but I would emphasize again that these are always freshly implanted cells (generally small numbers of cells) which have been allowed to come in contact with a very large amount of antibody. This is well reported in the literature.

**Feldman:** How about a normal tissue graft?

**Winn:** If we place Lewis rat skin on immunosuppressed mice, the grafts can be destroyed by injections of BN anti-Lewis alloantiserum. We also can destroy Lewis skin grafts on BN recipients with the same antiserum. These observations are highly reproducible, and it seems appropriate to consider why we can do this routinely, when others have found it difficult or impossible to influence the course of allografts of skin through the administration of alloantibodies. It turns out that, in our experimental systems, the grafts are resistant to both alloantisera and heterologous antisera until about the 9th day after transplantation. Rat skin allografts that had AgB antigens lacking in their hosts would be rejected or very badly damaged by this time. Thus, it is impossible to demonstrate the destructive effects of antiserum in recipients whose immune responses have not been suppressed.

We can also do this on rats. The answer is very simple. The grafts placed on these animals, mice or rats, are not susceptible to the effects of antibody for the first 8–10 days after transplantation, and first-set skin grafts in rats would be completely rejected by this time. So the susceptibility first appears at about day 9–11 and disappears after about 30–35 days. Therefore, the only way you could ever test these grafts, put antibody in during the sensitive period, is on a suppressed animal.

**H. T. Wepsic:** On the system in which you have both enhancing and cytotoxic “antibody,” have you attempted to separate this out to see if they are both in the same class?

**Winn:** No.

**J. Longstreth:** Do you know the mechanism of the cobra-venom factor on your cells?

**Winn:** I'm sure there are other people, and probably the Chairmen here, who are better qualified to answer that. Briefly, what is stated in the literature is that cobra venom activates C3 and then the complement system begins to act sequentially leading to depletion of C3 and other late-acting components.

**M. M. Sigel:** Would you kindly explain the effects of the chicken antirat serum which caused survival of your grafts in the presence of mouse antirat serum?

**Winn:** It's been stated, or at least rumored, that avian antibodies do not fix mammalian complements effectively, and the chicken antiserum was made with that in mind. If we use this chicken antiserum *in vitro*, we show that it fails

to activate complement from rabbits, guinea pigs, or mice. Furthermore, it will block the cytotoxic effects of other antibodies specific for rat cells.

**A. J. Girardi:** You showed a serum that was raised in a Lewis rat. You stated that it was cytotoxic for carcinoma and for an ascites cell, but that it enhanced the sarcoma *in vivo*. Is it cytotoxic for the sarcoma *in vitro*?

**Winn:** Yes. That is not the first time that has been noted. In fact, Hildemann and Bloom found the same thing with an apparently tumor-specific transplantation antigen and the corresponding antibody. They could detect cytotoxicity by the very sensitive technique of culturing the cells in the Falcon microtest plates. If they added tumor-specific antibody and complement to these cells, then the cells came off the plastic surface and apparently were destroyed. However, when this same antibody was injected into mice implanted with tumors, they consistently observed enhancement of the tumors.

**P. Perlmann:** I want to ask you about complement-dependent graft destruction. You said that you believed leukotactic factors were doing the job: Can you exclude that you are also dealing with an opsonic effect dependent on cytotoxic effect set in motion by C3?

**Winn:** That is possible with tumor cells but unlikely with the skin graft. I find it hard to see how cells can actually phagocytize a highly structured graft like that. It may be that they are attempting to and, in the process, are degranulating. The first thing one sees, at about 5 minutes, is margination of the granulocytes along the small vessel walls, and then the cells enter the interstitium. Very intense edema follows, and it is probably related to degranulation release of vasoactive substances.

With tumor cells it's quite likely that phagocytosis is involved, and certainly the people at Seattle, Dr. Russell Weiser and his group, have described the important role of macrophages in the destruction of ascites tumor cells.

**T. Borsos:** I have a comment on that particular point. It's a mistake to think of a bound C3 molecule as an opsonizing molecule. This is simply an operational definition. It's quite well demonstrated that the bound third component is making the cell more sticky, and doesn't necessarily imply opsonization.

So it's perfectly proper to think of bound C3 as making the tumors cells give off a single saying, “I am now available: Please do come and attack me.” What the ultimate attack will be—opsonization or just another form of kiss of death—we don't know yet; but let's not think of bound C3 only as an opsonizer.

**W. J. Martin:** An important distinction exists between your system and a tumor system. You have a vascularized skin graft, which may be rejected by antibodies reacting with endothelial cells rather than epidermal cells *per se*. Thus it's known that grafted kidneys can be rejected by antibody reacting with endothelial cells of the graft. Tumors, however, are vascularized by normal host blood vessels, and antibody-mediated tumor destruction could not occur via an antiendothelial cell reaction. Rather the antibodies would need to traverse the capillaries to reach and react



with the tumor. Can one extrapolate from your model to argue for antibody rejection of the parenchymal cells of a growing tumor?

**Winn:** Yes, I don't think there's any question of that. The speed of the reaction and the histologic observations that we have made are consistent only with the notion that the antibody is reacting, in the case of the skin grafts, with antigens on the cell membrane of vascular endothelium and that a large part of the damage we see is unquestionably due to infarction of the graft.

On the other hand, infarction must play some role in the destruction of the tumor graft. Getting back to what Dr. Borsos mentioned previously, ultimately we are interested in the effects of immune substances on vascularized established grafts.

But I do think that the weak point in extrapolating from skin to tumors—and maybe this is what you have in mind—is that it's difficult to infarct the entire tumor. For one thing, the cells are generally much less sensitive to ischemia, and only a very small fraction of the cells have to divide for the tumor to take off again.

So I don't think we can make a complete extrapolation. The main point I wanted to make with the data is simply that it seems to me, at least in the mouse, the traditional cytolytic mechanism that we look at *in vitro*, and which seems to require all—at least all of the known—components of complement, plays a much less important role than we would have thought *in vivo*.

**M. Takasugi:** Dr. Levy, have you typed tumor cells in culture and, if so, how does this typing compare with lymphocyte typing?

**N. Levy:** We have not done a full typing, as you have done with cultured tumor cells, but in every instance where we have looked at patients in whom we have either cultured fibroblasts or cultured tumor cells from patients on whom a full lymphocyte typing has been performed by standard techniques, we have seen complete correlation between the tumor cell or fibroblast typing and the lymphocyte data. In no instance have we failed to detect on tumor cells or fibroblasts antigens that were detected on lymphocytes.

**Question from the floor:** Would the cells be equally sensitive?

**Levy:** I can't say that they are equally sensitive. I can just say that Dr. D. B. Amos and his group can detect on lymphocytes a certain population of antigens, and we have detected the same antigens whenever we have tried with the appropriate monospecific isoantisera.

**J. E. Kennedy:** I think you said that human complement did not easily lyse human nucleated cells. If so, can you really hope to get lysis of human tumor cells *in vivo*?

**Levy:** Dr. Winn's point in this regard is well taken, that the cytotoxic endpoint we are looking at merely enables us to detect the interaction of complement with the antibody. It's possible that in the human system, if complement is important, it is acting not through the cytotoxic potential of the full complement sequence but rather through biologically active cleavage products of various components, such as chemotactic factors, anaphylatoxin, etc. The first thing we did, as a matter of fact, before we got involved with the system I've presented here, was to try different

human complement sources. We tried 15 different adult human complement sources and 9 different human cord complements. Each of these sera had normal hemolytic titers but failed to effect lysis of fibroblasts or tumor cells sensitized with monospecific anti-HL-A isoantisera or anti-tumor antoantisera.

**C. McKhann:** Some cells are notoriously resistant to the effects of antibody in complement. You showed dramatic effects of cytotoxic antibody on some cells with your manipulations of the complement system. Were these manipulations also effective on cells, like sarcoma cells, that are relatively resistant to cytotoxic antibody?

**Levy:** We have not tried mouse sarcoma cells nor have we tried enough cells to state that there is a population of cells resistant to cytotoxic antibodies. We have never found such a population and have tried human sarcoma cells as well as melanoma, breast, and colon tumor cells. Our primary experience is with brain tumors, however.

**McKhann:** Were any of these cells clearly resistant to more conventional techniques of cytotoxicity?

**Levy:** When you say resistant, if you are talking about being resistant to lysis by appropriate antibody and human complement, they all were.

However, if you talk about resistance to the appropriate antibody and rabbit or rat complement, we found complete correlation between the lysis of cells by rabbit or rat complement and lysis of cells with our dual reagent system. Our system, however, was free of background lysis of unsensitized cells by complement alone and also seemed to induce slightly more lysis of sensitized cells than either undiluted whole rat or rabbit serum.

**Borsos:** Any more questions of Dr. Levy?

**C. E. Mawas:** How do you assess the most efficient ratio of the two-component source of complement for one given cell? Are you using standard ratio of rat and human complement, or do you have to assess each time which is the most efficient complementation system for each given target cell?

**Levy:** Since we got no lysis of sensitized cells with human complement, comparison of our system with human complement was not essential. We do not attempt to optimize the procedure with each target cell system and don't know if this is necessary.

**S. Rosenberg:** Do you have any evidence that with your complementation system you can detect anything that cannot be detected by a conventional rabbit complement source specifically absorbed with the target cells?

**Levy:** No, we don't. Rabbit or rat serum can be effectively absorbed with the specific target cells to be employed. However, such absorptions require very large numbers of cultured cells, approximately  $10^8$  cells/ml of complement. A point I want to stress, however, is that absorption of complement with one target cell may not remove the xeno-antibody activity against other target cells. For instance, we have absorbed rat complement with glioma cells and removed background activity against glioma cells but not against sarcoma or melanoma cells. The beauty of our system is that it can be used on all target cells homologous to the first complement reagent and is thus ideal when one is studying several target cells and needs zero background lysis for best interpretation of his data.

# ***In Vitro* Methods for the Assessment of Cell-Mediated Tumor Immunity<sup>1, 2</sup>**

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**SUMMARY**—The advantages and limitations of some of the *in vitro* techniques used to detect cell-mediated immunity to tumor-specific antigens are outlined. These methods, although not originally devised for this purpose, have been adapted to measure the effect on tumor cells, the effect on specifically sensitized lymphocytes, and the effect their interactions exert on the migration of normal macrophages. These techniques have been used in many animal and/or human models.—*Natl Cancer Inst Monogr* 35: 21–26, 1972.

THIS ARTICLE is limited to a discussion of the *in vitro* techniques frequently used to detect cell-mediated immunity (CMI) to tumor-specific antigens (TSA). Since the vast literature available was recently reviewed by others (1–5), this brief survey considers only selected articles thought to be representative. The technical details relevant to the methodology, also adequately compiled (6), are not included.

Knowledge of the role of CMI in tumor immunology obviously has been advanced by *in vitro* techniques carefully devised to detect TSA. Most of the techniques now used were originally introduced to detect CMI to histocompatibility antigens. The similarity of target-effector cell interactions in transplantation and tumor immunology, therefore, demands that any *in vitro* test must conclusively demonstrate a specific reaction involving TSA. This is particularly important because a good complement of histocompatibility antigens is expressed on most tumor cells. The availability of in-

bred lines of animals has essentially eliminated this difficulty in experimental model systems. However, it still represents one of many problems encountered in human studies.

Several techniques based on the detachment of damaged cells from glass or plastic surfaces have been devised to detect CMI to TSA. Cells that normally grow firmly attached to surfaces adhere as long as they remain viable. When damaged, they detach and are destined for lysis. The detachment of tumor cells is taken as evidence of the cytotoxic effect of sensitized lymphoid cells. The most commonly reported technique based on this phenomenon is colony inhibition (2, 3, 7). Tumor cells prepared from trypsinized cultures are cultivated overnight to allow them to settle and attach to the culture vessel before cell suspensions prepared from lymph nodes, spleen, or peripheral blood are added. After several days' incubation, the number of colonies, each developing from an adherent cell, are counted. The number of colonies formed in the presence of immune lymphocytes is compared to that formed in the presence of control lymphocytes. These results are usually expressed as a percent reduction.

The colony-inhibition test has been used to detect CMI to a wide variety of tumor cells. The high ratio of lymphocytes to target cells which can be attained contributes to the marked sensitivity reported for this technique. The limitations of this test center about the target cells. The tumor must be propagated *in vitro*, and it has been reported

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ported that <25% of the human tumor explants have been successfully cultivated (8). A related problem is the plating efficiency. The density and distribution of seeded tumor cells affect the occurrence of a chance event leading to a sensitized lymphocyte encountering a target cell. Tumor lines with a high plating efficiency increase the probability of the chance event and affect any comparative studies on tumor lines of different plating efficiencies.

The subjectivity of scoring colonies, frequently mentioned as another limitation, for the most part has been avoided by a modification of the colony-inhibition assay (7,9,10). The assay is performed in small wells of plastic culture plates and has the advantage of requiring microliter quantities of materials. The principle of this technique is the same as that of colony inhibition except cells instead of colonies are counted (11,12). Results are expressed as the percentage of cells surviving in the wells to which immune cells were added as compared to the percentage of cells surviving in wells to which nonimmune cells were added.

The sensitivity of this modified test compares well with that of colony-inhibition assays. With this technique, nonspecific cytotoxicity is significant, particularly when leukocytes from peripheral blood are used as a source of effector cells. For example, the number of target cells surviving in control wells can increase twofold if granulocytes and monocytes are removed from peripheral blood leukocytes. This type of nonspecific cytotoxicity usually exceeds the cytotoxic effect attributed to immune lymphocytes.

Another modification of the colony-inhibition test involves the use of radioactive precursors for labeling the tumor cells. The tumor cells are labeled with either  $^3\text{H}$ -thymidine (13) or  $^{125}\text{I}$ -iododeoxyuridine (14), and both colony-inhibition and the microassay technique have been performed with labeled tumor cells. The radioactivity remaining in the culture vessels after incubation with lymphocytes is correlated with target cell survival. Results are expressed both as counts/minute and the percent loss of target cells calculated from the radioactive data. The sensitivity of the techniques apparently has not been affected by use of isotopically labeled target cells, but occasionally there is a high nonspecific loss of target cells in control cultures, particularly in experiments involving humans (13). This is due most likely to cytotoxicity associated

with mixing allogeneic cells rather than to spontaneous release of isotope. Out of necessity, normal lymphocytes prepared from healthy volunteers are used in control assays. Cellular interactions can occur under such allogeneic conditions and, although the mechanisms are poorly understood, nonspecific cytotoxicity has been observed. This could well account for the high control value, since care was taken in these studies to partially purify the lymphocytes prepared from peripheral blood.

Use of isotopically labeled target cells eliminates the tedium of visual enumeration of cells or colonies. In addition, it circumvents any criticism that cells present in the lymphoid suspension contribute to the adherent population. This is particularly valid, since reutilization of any released isotope has been virtually eliminated.

Many different culture conditions have been devised to evaluate the participation of nonspecific factors that are involved in these assays. Of these, tumor cells cultured with nonreactive lymphocytes constitute the preferred control. Such a control also takes into consideration any feeder effect lymphocytes may contribute to the tumor cells. In addition, any adhering cells or colonies arising from cells in the lymphocyte suspensions will be scored in both control and experimental cultures, assuming immune and nonimmune lymphocyte suspensions are identical. This last problem is of minor significance if the tumor cells or resulting colonies are morphologically unique and easily distinguished from normal cells.

The direct lysis of target cells can be detected by an *in vitro* assay based on a procedure used by Brunner *et al.* (15) for detecting CMI to histocompatibility antigens. This technique involves labeling the target cells with  $^{51}\text{Cr}$ . The lysis of the target cells by lymphoid cells is detected by determining the release of  $^{51}\text{Cr}$  into the supernatant fluid. An inverse correlation between the amount of free  $^{51}\text{Cr}$  and surviving target cells strongly indicates that this is a valid index of target cell lysis. This method has been adapted for detection of CMI to TSA in both animal (16,17) and human studies (18–20).

This technique has several advantages, most importantly the short period of time involved. After only 3 hours of incubation, significant lysis of tumor cells can be detected. In addition, tumor cells do not have to be cultivated before use nor must they adhere to surfaces, an absolute requirement for all

previous techniques. Because the target cells can be directly counted before addition of the lymphoid cells, exact ratios are known and controllable. Utilization of the free isotope is of no concern because, during the binding, the chromate is reduced and in this form no further binding is possible.

Although virtually any cell can be labeled with  $^{51}\text{Cr}$ , some types of tumor cells are resistant to lysis. This then restricts the general acceptance of this technique. The requirement for cell lysis may also be responsible for the difference in the sensitivity between this assay and those assays that rely on detachment of target cells from surfaces. The detachment of target cells most likely does not require lysis of the cells.

Changes in the metabolism of tumor cells induced by the cytotoxic effect of lymphocytes have also been used as a measure of CMI (21). The incorporation of radioactive precursors into cell products is the most common technique used in these types of assays. Inhibition of utilization of these precursors is taken as evidence that the tumor cells have been damaged by the immune lymphoid cells. The major limitation of these techniques is that the lymphoid cells can also incorporate the label. However, during the initial 24 hours of incubation of lymphocytes and target cells, most of the isotopically labeled thymidine is incorporated into tumor cells (22). This differential makes it possible to establish significant changes, but results are still difficult to evaluate.

*In vitro* techniques, not based on cytotoxic effects on target cells, have recently been used to detect TSA. They take advantage of the observation that specifically reactive lymphocytes, on interaction with antigen, undergo detectable morphological changes and/or elaborate active factors.

One method involves the transformation of lymphocytes. Lymphocytes from a sensitive donor, when combined with antigen in culture, transform into blastlike cells which then proliferate (23). The percentage of lymphoblast can be evaluated by a direct microscopic count, but the uptake of  $^3\text{H}$ -thymidine by proliferating cells is more commonly used as an index of lymphocyte transformation. TSA reportedly induce similar effects (24, 25).

Several factors must be closely controlled to interpret the results of lymphocyte transformation induced by TSA. For example, plant mitogens are much more potent in inducing blastogenesis than TSA, and these reagents should be avoided. Also

blastogenesis occurs when normal lymphocytes from two individuals with different histocompatibility antigens are cultured together (26). This last consideration is quite important in human studies, and the clearest results are invariably obtained when the patients' lymphocytes are cultured with autochthonous tumor cells. The lack of autochthonous tumor cells sometimes limited the collection of meaningful data during various clinical stages of disease. However, tumor cells now can be stored in the frozen state and used later with minimal loss of activity (25, 27). This should be a significant aid and eliminate the need to consider stimulation induced by allogeneic cells.

The uptake of  $^3\text{H}$ -thymidine by proliferating cells is particularly important if both the target and effector cells can incorporate the labeled precursor. In attempts to limit the incorporation of  $^3\text{H}$ -thymidine to only one cell type, target cells are inactivated by treatment with agents which eliminate or markedly reduce the target cells' ability to incorporate the labeled precursors. The procedures most commonly used to treat the target cells are use of mitomycin C or large doses of ionizing irradiation.

An additional point concerning the incorporation of  $^3\text{H}$ -thymidine by target cells involves the calculation of a reactivity index. This value is arrived at by determination of the ratio of counts/minute of cultures containing the active lymphocytes and mitomycin C-treated target cells to the counts/minute of cultures containing only mitomycin C-treated active lymphocyte. However, mitomycin C-treated target cells can incorporate detectable levels of  $^3\text{H}$ -thymidine, and this value is often excluded from the calculation of the reactivity index. If the counts/minute observed with mitomycin C-treated target cells are added to the counts/minute observed with mitomycin C-treated lymphocytes and this total value is used to determine the reactivity index, the value of the ratio is considerably lowered, even to the point of indicating no or marginal blastogenesis. Therefore, it is more meaningful to report the counts/minute data for all experimental and control cultures, if reactivity index values are to be evaluated.

Although the results obtained by culturing lymphocytes from sensitive hosts with antigen correlate well with other parameters of cellular immunity (28, 29), there is evidence that cells involved in humoral immunity are also capable of lymphocyte transformation (30, 31). Until these



problems have been resolved, the results obtained by lymphocyte transformation reactions must be limited to an index of recognition of antigen by lymphocytes. However, modern-day cancer immunology depends on the demonstration of antigenic differences between normal and tumor cells, and this technique is a sensitive assay for detection of these differences.

A second *in vitro* technique, not dependent on target lysis, involves the inhibition of macrophage migration (32-34). Macrophages, harvested from the peritoneal cavity of immunized donors and mixed with specific antigen before being pelleted in capillary tubes, do not migrate out onto the surface of the culture vessel to the same extent as do macrophages not mixed with antigen. The migration of macrophages is also not affected by incorporating antigens to which the donor was not immunized. It has been now conclusively demonstrated that the lymphocytes contained in peritoneal washings constitute the active component (34, 35). Only a few lymphocytes, about 2-3%, need be included with normal macrophage populations to demonstrate inhibition of migration of these normal macrophages.

Tumor cells have been shown to inhibit the migration of macrophages from immunized animals. The inhibition is markedly specific and has been reported with both intact tumor cells (36) as a source of antigen and soluble extracts of tumor cells (37-39). In addition, lymph node cultures of sensitized guinea pigs, exposed to a tumor extract, elaborate a soluble migration inhibition factor (MIF) which effectively inhibits the migration of normal peritoneal exudate cells (37). Although the assay for detection of MIF is not as sensitive as the direct assay technique, it is significant that these observations closely parallel the results obtained in systems involving soluble protein antigen (34, 35).

Use of soluble extracts has significant advantages, since this permits not only some degree of quantitation but also allows comparisons between various *in vivo* and *in vitro* techniques. By standardizing the tumor cell extract on the basis of protein content, it has been demonstrated that the inhibition of macrophage migration correlates well with lymphocyte transformation and the intensity of the skin reaction (38).

Similar studies in humans (40, 41) have been more difficult, mainly because of the lack of an

easily obtainable macrophage population. Cultured human lymphoid cells (42) and guinea-pig peritoneal exudate cells (43, 44) have been used as a source of target cells for detection of MIF produced by human peripheral blood cells. When peripheral blood cells are used in these assays, the complication that migration inhibition depends on the presence of neutrophils raises doubt as to the nature of the reaction. Buffy-coat cells devoid of neutrophils do not show specific inhibition of migration when soluble antigens are used (8, 45), and the possibility that cytotoxic antibody absorbed to neutrophils is involved is troublesome.

There seems to be little doubt that TSA can be demonstrated and that lymphoid cells can interact specifically with these antigens, whether membrane bound or soluble. Whether the same antigen is involved with all or some of these techniques is not clear and requires further investigation. In addition, more comparative studies and a means of quantitation to determine the correlation between these techniques are badly needed. Besides the interrelation of the different *in vitro* techniques, these various assays should relate to the *in vivo* situation if CMI is to be of any therapeutic or diagnostic significance. Some attempts have been made along these lines, but again quantitation is difficult, particularly since many *in vivo* parameters such as the tumor load the patient carries must also be considered.

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# **In Vivo Methods for the Assessment of Cell-Mediated Tumor Immunity<sup>1</sup>**

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**SUMMARY**—The most important expression of cell-mediated tumor immunity *in vivo* is resistance to tumor growth; other *in vivo* or *in vitro* methods to detect cell-mediated immunity do not always reliably measure tumor resistance. Transplantation methods can detect resistance to tumor growth. Their interpretation is critically dependent on the controls used. They are poorly quantitative, complex, and cumbersome. The value of transplantation methods as models of the immunobiology of human cancer is limited by 1) differences between transplanted tumors and primary tumors, 2) differences between experimentally induced tumors and naturally occurring tumors, and 3) differences between species. In new *in vivo* methods, it is desirable to strive for better quantitation, standardized materials, and means to study component processes in isolation or under controlled interaction and to distinguish qualitative from quantitative effects and cytotoxic from cytostatic effects.—*Natl Cancer Inst Monogr* 35: 27–35, 1972.

THE DISTINCTION between antibody-mediated and cell-mediated immune responses may not be as clear as it seems. It is possible to isolate antibodies and to study their activities in cell-free systems. However, it may not be possible to study cellular function in the strict absence of extracellular antibody, since lymphoid cells produce antibodies. Furthermore, some types of lymphoid cells may function through antibody-like receptors on their surfaces. So cell-mediated immunity may mean immune reactions in which antibody is not detectable, or, in more general terms, reactions which can be transferred to nonimmune, compatible recipients by grafts of living lymphoid cells, but not by cell-free antibody. Such cell-mediated immune reactions are exemplified *in vivo* by delayed cutaneous hypersensitivity reactions, by some

autoimmune processes, and by the immune rejection of living tissue grafts.

Without tracing the historic developments, suffice it to say that immune reactions against syngeneic or autologous neoplasms are possible. Several types of tumor-specific immunity may be potentially beneficial, but the emphasis here is on that sort of immunity tending to protect an individual from the growth of his own tumor. The mechanism of such tumor resistance may be similar to that for the cell-mediated rejection of non-neoplastic tissues. This paper describes the basic methods for the study of such resistance in syngeneic, transplanted tumor systems, considers advantages and disadvantages of those methods, evaluates the relevance of transplantation methods to the problem of host–primary tumor interactions, and suggests some potentially fruitful areas for technologic developments in the study of cell-mediated tumor immunity *in vivo*.

Tumor immunity seems to be a special case of allograft-rejection immunity. However, because of the specificity, strength, and distribution of the specific tumor-rejection antigens, alloimmune reactions are poor models for the study of immune

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tumor resistance. Study of immunity to tumors in allogeneic or randombred systems may be informative concerning alloimmunity, but not concerning tumor-specific immunity. The latter process must be studied in the primary host, or in animals histocompatible with the animal in which the primary tumor developed. Comments here are restricted as much as possible to such "tumor-specific" studies.

## TRANSPLANTATION METHODS

The initial graft of a tumor to a normal, syngeneic recipient usually produces progressive, lethal growth, with little or no evidence of immune resistance by the host. This could be because no immunity develops, or because the immunity that develops is too little or too late. That the latter is frequently the case can be seen by a simple test. Growth of a tumor inoculum in normal animals is compared with that of a similar inoculum in animals prevented from developing any immune resistance by prior immunosuppressive treatment (1). Such a comparison may reveal a partially effective tumor resistance in the normal animals, presumably a result of the development of a primary immune response. This test is greatly influenced by the fact that, during the time required for the induction of the primary response, the cells which must be rejected have the opportunity to become physiologically established in the host and begin their neoplastic growth behavior. Thus the test is sensitive to the effects of tumor growth potential—early, rapid growth could obliterate the inhibitory effect of a developing immune response.

The latter problem can be minimized by study of the resistance of the more rapidly developing secondary, or anamnestic, response. This is done by determining the resistance of previously immunized animals to tumor growth. The problem here is immunizing the animals to the tumor without killing them with the tumor. Dead and disrupted tumor cells usually do not produce resistance to tumor growth. There are several methods to safely immunize with viable tumor cells. For reasons not entirely clear, many tumors will not grow in normal syngeneic animals after injection of individual tumor cells or a few tumor cells; frequently the threshold cell dose for growth is quite high. If animals receive injections of subthreshold numbers of tumor cells, they may develop immunity to that

tumor (2). Tumors exposed to X radiation or to other cell poisons may be unable to undergo mitosis, although they maintain most of their metabolic and structural integrity. Such cells are usually immunogenic (3); however, the treatment may alter the antigenic characteristics of the cells. Finally, if animals are cured by ligation (4) or surgery (5) after a period of temporary tumor growth, they may develop specific tumor immunity.

Immunity in animals so treated is evidenced by their resistance to growth of a challenge injection of viable tumor cells. However, not all that retards tumor growth is tumor-specific immunity (nor is all that accelerates tumor growth tumor enhancement in the immunologic sense). Therefore, valid interpretation is impossible unless challenge tumor growth in immunized animals is compared with challenge tumor growth in appropriate control animals. The nature of the control groups critically affects the resulting conclusions. Without control comparison, treatment effects cannot be distinguished from inoculum failure. The use of untreated controls allows the difference observed to be attributed to the treatment given. If the controls are pretreated by exposure to various syngeneic tissues—normal adult tissues, unrelated tumor tissue (5), or embryonic tissue (6)—information is gained concerning the specificity of the resistance. As before, growth of the challenge inoculum in the nonimmune or control animals would reflect the influence of a primary immune response, while that in the preimmunized animals would reflect the influence of a secondary response. Exposure of the animals to sublethal, immunosuppressive radiation just before the challenge injections (7) would allow a comparison of the radioresistant secondary response in the experimental animals with no immune response in the nonimmunized controls.

## Quantitation of Transplantation Methods

Means to evaluate the degree of immune resistance are indirect and semiquantitative at best. One approach is to assess the effect of immunity on growth behavior of the challenge tumor inoculum. Resistance might be expressed in any of 4 variables of tumor growth: 1) a lower incidence of tumor takes, 2) delayed appearance of tumors, 3) slower growth rate of the tumors, or 4) regression of tumors after temporary growth. Of course, tumor rejection is the most desirable result, and all-or-none effects

are easy to evaluate. However, immune responses to tumor-specific antigens are frequently weak, so the use of only one criterion, tumor incidence, may be ignoring a portion of the data available. Another approach to quantitation of the strength of the immune resistance is to compare, by use of several doses of tumor cells for challenge, the minimum number of cells that will grow progressively in the control and experimental groups (1). The difference in threshold doses is an estimate of the number of tumor cells killed by the immunity. Unfortunately, neither procedure directly measures the number of cells killed or distinguishes between cytotoxic and cytostatic effects.

### Limitations of Transplantation Methods

The semiquantitative nature of the *in vivo* transplantation tests is one of their major limitations. Another problem is the complexity of the system, involving the interplay of many immune and nonimmune processes. These may be divided conceptually into 3 major levels: 1) induction of the immune response, or immunogenicity; 2) host processes; and 3) the effectiveness of a given immune response in controlling tumor growth. The host-response variables can be standardized by the use of similarly treated, syngeneic animals, but they remain complex. With a few exceptions, the components of the other 2 categories are difficult to distinguish by use of the transplantation methods. Thus, a weak or absent resistance to tumor growth could reflect poor immunization due to paucity, distribution, coating, or size of antigenic determinants or to lack of adequate carrier or adjuvant properties in the preparation; the immunizing preparation may sensitize the host but produce a qualitatively or quantitatively ineffective response; or the growing, target tumor cells may differ in their sensitivity to immune destruction and in their growth behavior. An integral part of this problem is the fact that there are virtually no standard reagents for reference. Tumor cells by their nature are quite variable, and the use of such variable populations as both immunogen and target in these tests is a little like determining the distance between 2 points, both of which are in random motion. In addition to these interpretative, theoretical, conceptual problems, the *in vivo* tests have practical limitations, including the time required to conduct and evaluate the test and the

large numbers of animals required to obtain quantitative data.

### DELAYED CUTANEOUS HYPERSENSITIVITY

Other methods are available to assess cell-mediated tumor immunity *in vivo*. They minimize some of these problems but introduce limitations of their own. As was mentioned earlier, cell-mediated immunity *in vivo* can be expressed by delayed cutaneous hypersensitivity as well as by tumor rejection. This has been most useful in the guinea pig. If immunized guinea pigs are challenged with living tumor cells, they usually develop a typical, delayed cutaneous hypersensitivity reaction at the challenge site and prevent growth of the tumor cells (8). Control animals do not develop the skin reaction or reject the tumor. Since delayed cutaneous hypersensitivity reactions do not depend on tumor growth for their evaluation, this technique has the advantages of rapidity and of application in the study of non-neoplastic, irradiated, or non-living preparations.

Delayed cutaneous hypersensitivity reactions in other experimental species have been more difficult to study. In sensitized mice, they can be detected by the measurement of footpad swelling after injection of antigens. However, even in the guinea pig, the ideal animal for study of delayed hypersensitivity reactions, there is not a 1:1 correlation between delayed skin reactions and tumor rejection. Some tumors can produce tumor-rejection immunity without the development of grossly detectable, classical, delayed cutaneous hypersensitivity at the site of challenge (9). Furthermore, delayed hypersensitivity is still a complex process.

### PASSIVE IMMUNIZATION METHODS

Some components of the host response can be distinguished and studied by passive immunization techniques, wherein the test animal is sensitized not by exposure to the antigen but by transfer of lymphoid cells from an immune donor. In a local transfer method, tumor cells and lymphoid cells are first mixed *in vitro* and then injected into a histocompatible, nonimmune recipient (10). If the lymphoid cells are from a nonimmune donor, the injection site does not develop a delayed reaction and the tumor will grow progressively. The proper lymphoid cells from an immune donor will initiate



TABLE 1.—Comparison of immunity to line-10 tumor induced by various methods (17)

Method of immunization	Challenge—10 <sup>6</sup> living line-10 cells (21 days after immunization)	
	Delayed cutaneous hypersensitivity reactions* (24 hr after challenge)	Mean tumor diameter (mm) ± SEM† (19 days after challenge)
10 <sup>3</sup> line-10 cells	0, 0, 0, 0, 0, +	11.9 ± 0.3
10 <sup>6</sup> line-10 cells—excision day 8	0, 0, 0, 0, 0, 0	12.2 ± 0.3
10 <sup>6</sup> line-10 cells—excision day 15	0, 0, 0, 0, 0, 0	11.2 ± 0.6
1.5 × 10 <sup>6</sup> irradiated line-10 cells	0, 0, 0, 0, 0, 0	11.5 ± 0.3
1.5 × 10 <sup>6</sup> irradiated line-10 cells and 6 × 10 <sup>6</sup> BCG	+++ , +++ , +++ , +++ , + , +	0.0
6 × 10 <sup>6</sup> BCG	0, 0, 0, 0, 0, 0	13.0 ± 0.4

\* Skin reactions were graded on a scale 0-++++: 0: no erythema; +: pink; ++: bright pink; +++: bright pink with pale ischemic center; and ++++: bright pink with central necrosis.

† Standard error of the mean.

a typical, delayed cutaneous hypersensitivity reaction at the injection site (11), and the tumor will fail to grow. Another method involves systemic transfer of the immune cells before injection of the challenge inoculum (12). These passive transfer techniques may be used to study the actions and interactions of some lymphoid cell subpopulations by the injection of purified cell populations or controlled mixtures of them. The participation of the recipient can be diminished by sublethal irradiation before the transfer procedure (1), and the resultant lymphocytopenia probably promotes rapid repopulation by the grafted cells. However, radioresistant processes in the recipient may influence the outcome.

## MODEL BUILDING

The topic of this Conference is the immunology of carcinogenesis. From that, I infer considerations of immune interactions during the development of primary tumors. The transplantation methods I have described have value primarily as models of naturally occurring, *in situ* neoplasms. There are at least 3 model-object relationships which should be kept in mind: 1) transplantation methods as models of *in situ* primary tumors; 2) experimentally induced tumors as models of naturally occurring tumors; and 3) animal studies as models of immune processes in human cancer. There are several obvious differences between transplantation systems and primary tumors. The host of a primary tumor is immunized, if at all, during the development of his tumor *in situ* from a single, transformed cell with

little cell death or adjacent tissue damage. In contrast, transplantation methods involve the introduction of large numbers of cells, with attendant damage and death of both host and donor cells; they are usually applied only to certain convenient tissue sites. Assessment of immunity in the primary host suffers from the same limitations of quantitation, complexity, and variability discussed for the transplantation models. Any challenge procedure may alter as well as measure the host-primary tumor relationships. Presence or absence of the primary tumor will alter the results of a challenge procedure. Transplanted tumors may have undergone antigenic drift or have become infected with antigen-inducing passenger viruses. Finally, injected cells have a radically different physiologic relationship to the host than do the cells of an *in situ* lesion, as is suggested by the experimental system called concomitant immunity (13). Likewise, experimental methods of tumor induction may be poor immunologic models of naturally occurring tumors, since experimental carcinogens frequently interfere with host immune reactivity (14) and since many induced tumors have stronger tumor-rejection antigens than are commonly found among naturally occurring tumors (15, 16). Some insight into the possible problems of using animals as models for human disease can be gained by consideration of the degree of variability among different experimental species. For example, guinea pigs and mice differ in their response to the adjuvant properties of bacillus Calmette-Guérin (BCG) for tumor immunity. Table 1 shows the results of an experiment in guinea pigs with a

transplantable, chemically induced hepatoma. Three standard methods of immunization with this tumor—subthreshold tumor cell dose, excision after temporary tumor growth, or injection of irradiated tumor cells—produced neither delayed cutaneous hypersensitivity nor tumor-rejection immunity to challenge with living tumor cells. However, strong immunity of both types was observed in animals given a mixture of irradiated tumor cells and living BCG (17). Table 2 shows the results of similar tests with 4 different syngeneic sarcomas in mice. The first tumor produced resistance to challenge, but pretreatment with the other 3 tumors did not produce significant protection against tumor challenge. When the pretreatment consisted of the injection of a mixture of tumor cells and living BCG, as described for the guinea pig experiment, immunity was not augmented for the 3 nonantigenic tumors, and immunization by the antigenic tumor was prevented. This system has been varied in several respects, and as yet we have not obtained augmentation of tumor-specific immunity in mice with BCG, whereas we have consistently obtained such an effect in guinea pigs. The problem of extrapolating results of animal studies to human studies is further complicated by difficulty in doing the type of direct comparison just described for animals. It may not be possible to decide whether an animal model is an accurate replica of clinical problems in man.

This consideration of the types of limitations inherent in model building is not intended to discourage the use of model systems in tumor immunity. On the contrary, new models are needed that closely approximate the ultimate objective or that permit study of the model-object discrepancies. Unless such discrepancies are kept in mind, there is always a danger that the model will become the object of study.

Obviously the methods now generally available for the *in vivo* assessment of cell-mediated tumor immunity leave much to be desired. There is every reason to believe that new techniques will be developed. Some problems have been studied by refined methods *in vitro*, but *in vivo* parallels are needed; there are yet other problems to be resolved either *in vivo* or *in vitro*. The following areas might prove fruitful for technologic progress in *in vivo* study of tumor immunity: 1) the ability to distinguish and evaluate cytotoxic and cytostatic effects of immunity; 2) development of standardized

reference reagents, *e.g.*, soluble tumor antigens; 3) more sensitive means to detect tumor immunity;

TABLE 2.—Effect of BCG on immunogenicity of murine tumors

Tumor line	Incidence of challenge tumors in animals immunized with:		
	BCG intradermally	BCG intradermally; tumor growth and excision	Tumor-BCG mixture
1038	13/19	2/18	11/20
1714	8/9	4/9	6/9
1769	10/10	7/10	9/10
1647	10/10	9/10	10/10

4) more reliable means to quantitate tumor immunity; and 5) better means to reduce complexity, so individual components of the tumor-immunity process can be studied independently and in controlled combination. *In vitro* technology has made especially useful contributions in the latter 2 areas, by the use of radioactive labels to quantitate results and by the ability to isolate and manipulate reactants in the culture vessel. These tools have not been widely applied to *in vivo* systems. Some preliminary studies by Dr. John Kreider represent a step in this direction (18). This work involved what I will call an *intra vivo* system—the use of Millipore diffusion chambers cultured in the peritoneal cavity as a confined culture vessel. Tumor cells were prelabeled with  $^{125}\text{I}$ -5-iodo-2'-deoxyuridine, either *in vivo* or *in vitro*. They were then mixed with normal or with immune lymphoid cells and cultured *intra vivo* 5 days. Residual radioactivity of the chamber contents was ascertained in a gamma counter; representative results are shown in table 3. Tumor cells alone retained approximately 50% of the radioactivity in the initial inoculum. That value was not affected by the addition of normal spleen cells in a 10:1 spleen-to-target ratio. Chambers receiving heat-killed labeled tumor cells retained only 1–2% of the label. When tumor cells were cultured with spleen cells from immunized donors, residual radioactivity amounted to 10% of original. In these initial studies, an allogeneic combination was used, so the value of this test for study of tumor-specific immunity remains to be established. The method offers the prospect of improved quantitation and of *in vivo* isolation of reactants from cellular



TABLE 3.—Immune spleen cell cytotoxicity of tumor cells in diffusion chambers

Contents	cpm/Chamber ± SEM	Percent of original*
5 × 10 <sup>4</sup> tumor cells	11,920 ± 1,157	49
5 × 10 <sup>4</sup> tumor cells and 5 × 10 <sup>6</sup> normal spleen cells	11,310 ± 1,786	46
5 × 10 <sup>4</sup> heat-killed tumor cells	357 ± 52	2
5 × 10 <sup>4</sup> tumor cells and 5 × 10 <sup>6</sup> immune spleen cells	2,252 ± 640	9

\* 24,488 cpm/5 × 10<sup>4</sup> tumor cells on day 0; % of original =  $\frac{\text{cpm/chamber day 5}}{\text{cpm/chamber day 0}} \times 100$ .

host influences. As with the other systems described, it has limitations in its relevance as a model of immune resistance to naturally occurring tumors. In this case, of particular concern are the influence of the radiolabel on the tumor cells, the effect of the diffusion chamber environment, and the technical problem that not all the cells are labeled. The materials may be available to devise yet other methods for *in vivo* or *intra vivo* analysis of cell-mediated tumor immunity.

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## GENERAL DISCUSSION—PART 1

**H. J. Rapp:** It looks as if we are all fired up, and since one of the major purposes of my summary was to get you fired up, I don't think I have to do that. Why don't we go right now into the general discussion.

**P. Koldovsky:** I was prepared for the quick technical question, but since I am starting the discussion I can do even that. An important question has been discussed: What is the difference between immunization with live tumor, and what actually happened? We start to fractionate the cell, to extract the tumor-specific transplantation antigen (TSTA). Most of us agree that immunization with living tumor is more effective than the latter one. It may be for several reasons. First, living tumor cells repopulate and so it is a continuous supply of antigenic stimulation. Second, every procedure of extracting can damage the TSTA as well. For example, attempts to extract and define normal transplantation antigen were unsuccessful. Maybe we should think that TSTA is a dynamic status on (in) the cell membrane which is functionable only in an intact membrane and/or in contact (conglomerate) with a functioning (living) cell membrane.

**Rapp:** So the question is: Why do living tumor cells work so well and dead ones work so poorly? I think you've given us a few ideas. Dr. Bartlett, would you like to say anything about that?

**G. L. Bartlett:** Not much! I think that's an excellent and a very burning question. Work in our laboratory, which Dr. Leonard will describe, has led to isolation of a soluble tumor-specific antigen from some of the guinea pig tumors we have been studying. They are less immunogenic than the living cells. It is not simply a quantitative difference; this just is one type of problem that we may not yet have the techniques to look at. That is one of the challenges.

**R. W. Baldwin:** We have been studying, with one of my past colleagues, Dr. M. Moore, who is also here, the immunogenicity of subcellular fractions from a variety of chemically induced tumors, and we have in one particular example studied in some detail the immune parameters. With this hepatoma system (D23), we have methods that allow us to take the cell apart, and so isolate immunogenic plasma membrane fractions. We can solubilize the plasma membrane and get a purified tumor-specific antigen which is immunogenic *in vivo*. By that I mean that, if you inject either the membrane or the soluble antigen, you will produce tumor-specific antibody response and a very weak lymphocyte-mediated response. When you now look at the function of the tumor-specific antibody, it is cytotoxic in the presence of complement, but in the absence of complement it is highly blocking against sensitized lymphocytes and the overall response is nonprotective against a subsequent challenge with viable hepatoma cells.

A second effect of membrane immunization, where the main response is the production of tumor-specific antibody, is the development of a state of unresponsiveness.

If you now try to reimmunize those animals with irradiated, intact hepatoma cells, which are normally immunogenic, you don't get a protective response.

So what I'm trying to say is that in certain systems—and I know Dr. Rapp has had quite a different experience in the guinea pig—the subcellular fractions are not immunogenic in a protective capacity; this really needs to be worked on. The question we would put to the cellular immunologists is: Why are these isolated antigens processed in a different fashion to the intact cells?

**E. J. Leonard:** I will present in my paper a figure which shows that the KCl extract of hepatoma cells, emulsified in Freund's adjuvant, protects guinea pigs against subsequent challenge with live tumor cells. This doesn't really get at the quantitative question as to whether live cells or intact cells are as good as soluble extract, and that's a rather difficult question to answer.

**W. J. Martin:** As a comment on Dr. Nordin's paper, it is important to consider the possibility that the various assays of cell-mediated immunity detect different cell types which may have quite distinctive *in vivo* biologic function. A most important distinction may be between effector and regulatory cells. Effector cells are those whose primary *in vivo* function is to locate and destroy target tumor cells. Regulatory cells are specific for the tumor antigens but are postulated to function in regulating, either promoting or suppressing, the development of effector cells.

Among the lymphoid effector cells, we can perhaps make a further distinction between the type of cytotoxic cell detected in the 4-hour  $^{51}\text{Cr}$ -release assay of Brunner *et al.* and the colony inhibition and 24-hour microcytotoxicity assay used by the Hellströms. In a number of situations, the cells mediating the 4-hour assay are absent but colony inhibition is present in pregnancy. The next distinction among effector cells might be between macrophages and lymphoid cells. It is intriguing that a macrophage-inhibitory factor can be obtained from the amoeboid cells of sea stars (Prendergast). Perhaps a primitive defense mechanism mediated by macrophages may be present throughout phylogeny. This mechanism may certainly be active in tumor-bearing animals. This type of defense mechanism might differ from the highly specialized adaptive immune response, but in potency, specificity, and ability to cope with tumor challenge in different sites, *e.g.*, whether the tumor cells are localized in the skin or disseminated within various organs. Next it is reasonable to postulate the presence of regulatory cells. Very little is known about the cellular events involved in the generation of effector cells. By analogy with the cellular interaction required in the production of some humoral responses, we can envision the necessity of having several cell types to generate effector cells. For example, perhaps the cells which respond to tumor by blastogenesis *in vitro* may function *in vivo* not as an effector cell but rather as a cell which regulates the development of effector cells.

I have two additional comments. You mentioned that some cells did not appear to be lysed by cytotoxic lymphoid cells. A technical problem of the 4-hour assay is that, if one wishes to study adherent cell lines, one has to trypsinize the cells before the assay. Trypsinization may cause a loss of the tumor-associated antigen and apparent insensitivity to



lysis. However, if one cultures the cells in the individual test dishes for a 15-hour period after trypsinization and then labels the cells with  $^{51}\text{Cr}$ , in general the cells are now susceptible to lysis in a 4-hour lymphocytotoxicity assay.

The final point relates to the problem of why live cells are immunogenic for cell-mediated immunity but dead-cell antigens are nonimmunogenic unless incorporated in complete Freund's adjuvant. The need for Freund's adjuvant to elicit cell-mediated immunity by nonviable antigen emphasizes the distinction between antigenicity and immunogenicity. Especially with thymus-derived cells, activation may involve a 2-step process. It may therefore be difficult to detect antigens with the use of cell-mediated assays. Tumor antigens may be present on other cell types, or on a subcellular component. But, unless they happen to exist in a form that is appropriately immunogenic for the *in vitro* assay under study, their presence may not be detected. Antibody-binding assays are much more reliable for the detection of shared antigens.

**N. Levy:** Perhaps related to the differences in reactivity between living and killed cells are some data we have in our human brain tumor system. Based on *in vitro* cross-reactivity patterns, we have evidence for different antigenic determinants or, perhaps, different forms of the same determinant, involved in blocking as in cell-mediated reactivity.

**A. LoBuglio:** I have a specific question for Dr. Nordin regarding the migration-inhibition factor (MIF) assay. One of the questions from people who are trying to approach MIF assays is what cell line to use as the migrating cell. Much work has been done with guinea pig peritoneal macrophages to characterize this factor and to demonstrate its specificity.

On the other hand, we find the literature replete with a wide variety of migrating cells to be used to assay MIF, including cultured lymphoblasts, granulocytes, whole blood leukocytes, mononuclear cell preparations, and even tumor cell lines. I was wondering what your evaluation is of whether all these migrating cell lines can be equated with the guinea pig peritoneal macrophage data. If not, then how should we interpret migration inhibition of these other cell lines in terms of T-cell or cellular immune activity?

**A. Nordin:** I am not the one to comment on the relative ability of different cell types to migrate, because most of what I was exposed to was the macrophage migration involving guinea pig peritoneal exudate cells. I think the point that I was concerned with was the difficulty in interpreting the data on macrophage migration, when human buffy-coat cells are involved. Now whether such cultured cells can be used instead of macrophages I really don't know.

**Levy:** In the human, where the source of migrating cells poses a considerable problem, chimpanzee macrophages appear to work quite well.

**J. C. Kennedy:** What type of evidence is there that specific antibodies as such can cause rejection of an established tumor? Transplanted histoincompatible tissue is not really a suitable model system, because in that model the blood vessel endothelium is from the donor rather than from the host, whereas in a tumor the blood vessels are host blood vessels. An attack directed against histoincompatible endothelium could lead to rejection of a graft by

damaging and plugging the capillaries, but such a mechanism could not operate to cause tumor rejection.

**H. J. Winn:** That's right, and the point was brought up earlier that a great deal of the tissue damage in vascularized graft is attributable to infarction and ischemia.

On the other hand, grafts of dissociated epidermal cells, which become attached to the graft bed and make use of host vessels, are rejected by mechanisms similar to, or identical with, those involved in the rejection of full-thickness skin graft.

The only evidence that I know of for the destruction of an established and vascularized tumor comes from some work reported by Dr. Fefer from Seattle. I am not familiar with the details of that work, but I am under the impression that he secured in a few instances the reduction—or total destruction—of tumors by injecting humoral antibodies, specific for a tumor antigen.

**Rapp:** Yes, I believe that's correct. Does anybody want to comment on Dr. Fefer's work? I think this was a combination, wasn't it, of chemotherapy and passive transfer?

**Winn:** Yes, I think a Moloney virus-induced lymphosarcoma was used.

**Rapp:** Yes, that's right. Dr. Weiss, did you have a comment on this point?

**D. W. Weiss:** I would like to return for one moment to the question of the relative immunogenicity of living and dead tumor cells. It may be useful to bear in mind here the parallel model of microbial antigens. It is well known to us that intact microorganism will evoke immune responses to specific antigens within the microbial cell which are very different than if one employs the *isolated* microbial substances as antigens. Yet there is evidence, in many of these instances, that the *specificity* of the immunologic reactivity is identical. One may be seeing then an intrinsic "adjuvanticity" of other, perhaps nonantigenic, components or of components with other antigenicity, which can modulate the nature of the immune response to a given antigen.

It would be well to bear this in mind with regard to tumor cells as well and not to conclude too readily that one deals with *different* antigens or with qualitative changes in given antigens when one compares living with nonliving tissue. Instead, the possibility should be seriously considered that there is a modulating activity of other materials within living, intact tumor cells which can *direct* the immune response to the same antigens into certain channels.

**D. S. Yohn:** The comments I would like to make are relevant to the problem of whether soluble tumor-cell membrane materials can be used as effective immunogens. We have been studying an adenovirus-12 tumor system, a virus-induced tumor system that does not shed virus. We are not working with virus structural antigens here but with specific tumor-membrane antigens.

These studies were carried out in conjunction with Dr. A. Hollinshead in an inbred hamster system, line PD-4. In this system, we extracted from the tumor membranes a specific soluble glycoprotein which had an approximate molecular weight of 70,000. A dose of 90  $\mu\text{g}$  of this material, given intraperitoneally without the addition of mycobacteria to it, can immunize hamsters and protect

them against challenge of  $10^6$  tumor cells—a dose which induces tumors in 90–100% of sham-immunized hamsters.

One can also add mycobacteria, and it also works that way.

The interesting thing about the model is that the antibodies induced are not cytotoxic and have certain properties that resemble blocking antibodies. But nonetheless the animal is protected.

An important point concerns the methods used to solubilize the membranes. The procedure employed involves

freezing and thawing and light sonication of the membranes. Other methods, such as KCl extraction or enzymatic release, have not been evaluated. The various methods being used to solubilize immunogenic materials from membranes should be evaluated thoroughly in several different animal systems before we can recommend specific immunotherapeutic applications on a broad scale.

**Rapp:** Before we proceed further, Dr. Witz and Dr. Barth would like to tell us something about some methods they are developing.





## Tumor-Associated Immunoglobulins: Nature of the Association<sup>1, 2</sup>

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IT WAS shown that mouse tumor cells are coated *in vivo* with immunoglobulin (Ig), primarily of the IgG2 class (1). *In vivo* coating can be demonstrated either by immunochemical analyses of acid eluates from tumor cells or from membrane-rich fractions or by the ability of freshly harvested tumor cells to fix radiolabeled reagents directed against mouse Ig (2). Tumor growth was sometimes enhanced *in vivo* when tumor cells were incubated with IgG2-containing eluates before the cells were inoculated into syngeneic recipients (3).

When Ig-coated tumor cells were transferred to *in vitro* cultures, they rapidly shed some of their Ig coat. This process seemed to require conditions favoring active cellular metabolism. Some of the Ig in the culture medium apparently could be re-fixed by the cells. Furthermore, exchange of Ig from the surrounding of the cell with Ig on the cell surface may occur under natural conditions.

The possibility that tumor cells fix Ig by non-immunologic mechanisms was tested. IgG2, isolated from sera and ascitic fluids of mice bearing a  $\gamma$ 2-producing plasma cell tumor, was radioiodinated and incubated with tumor cells. The cells were able to fix the Ig, although it was probably not an antitumor antibody. Most of the  $\gamma$ 2 fixation could be inhibited by the addition of unlabeled, isolated IgG2 or by the addition of sera from normal mice but was not inhibited by the addition of unlabeled, isolated IgG1. The results suggest that the Ig coat of tumor cells may be partly composed of antitumor antibodies and partly of Ig fixed by tumor cells "nonspecifically."

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<sup>1</sup> Presented at Conference on Immunology of Carcinogenesis, held by the Oak Ridge National Laboratory at Gatlinburg, Tenn., May 8-11, 1972.

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<sup>3</sup> Karolinska Institutet.



# A New Radioisotopic Microcytotoxicity Assay of Cellular Immunity Utilizing Technetium-99m<sup>1, 2</sup>

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PREVIOUSLY REPORTED *in vitro* radioisotopic assays of cellular and humoral antibody-mediated immunity have employed nuclides or compounds which could be degraded, released, and/or reutilized during the course of an experiment (1-10). This could be a serious disadvantage in tests where target cells and lymphocytes are admixed with one another for periods of time greater than a few hours.

We have developed a new radioisotopic microassay utilizing technetium-99m (<sup>99m</sup>Tc), a nuclide which is bound to cells by reductive treatment and does not appear to be reutilized following release (11). The development of immunity in C57BL/6 mice (*H-2<sup>b</sup>*) to Sarcoma I (Sa-I), a fibrosarcoma of strain A/J (*H-2<sup>a</sup>*) origin, has been assessed *in vitro* by the <sup>99m</sup>Tc assay. The data obtained have been compared with the tritiated thymidine (<sup>3</sup>H-TDR) (9) and <sup>125</sup>I-5-iodo-2'-deoxyuridine (<sup>125</sup>I-IUDR) isotopic assays (10) and with a microcytotoxicity assay based on the visual enumeration of surviving cells (12, 13).

## MATERIALS AND METHODS

C57BL/6 mice were inoculated subcutaneously in the right subscapular region with 0.1 cc of minced Sa-I tissue (approximately  $20 \times 10^6$  cells) on day 0. Tumor growth was subsequently followed at 3-day intervals. Length (*c*), width (*d*), and

height (*e*) of tumors were measured with calipers, and volumes (*V*) were calculated according to the formula  $V = 0.52 \text{ } cde$  (14).

<sup>99m</sup>Tc is a high-specific activity metastable isotope (half-life = 6.0 hr) obtained as sodium pertechnetate ( $\text{Na}^{99m}\text{TcO}_4$ ) from a molybdenum-99 generator. Target Sa-I cells were labeled by the addition of 10 mc of <sup>99m</sup>Tc to 10 ml of Hanks' balanced salt solution (HBSS) containing  $10^7$  cells; this mixture was then incubated for 30 minutes at 37°C and was occasionally shaken. After incubation, 0.4 ml of a freshly prepared 0.1% solution of  $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$  was added dropwise to reduce the <sup>99m</sup>Tc from a valence state of +7 to +2 (15), and the cell suspension was washed 3 times with HBSS to remove unbound isotope.

The <sup>99m</sup>Tc-labeled Sa-I cells were resuspended in HBSS, viability was determined by trypan-blue exclusion (16), and the final concentration was adjusted to  $10^6$  living cells/ml. Two-tenths ml of Eagle's minimal essential medium, supplemented with 10% newborn calf serum, was added to each of 96 wells of a Falcon Microtest II<sup>R</sup> plate (#3040). With the use of a 500- $\mu$ l Hamilton syringe having a repeating dispenser, 10  $\mu$ l volumes (10,000 viable cells) were dispensed into each well.

Cellular and humoral antibody-mediated immunity of C57BL/6 mice to Sa-I was determined 3, 7, 10, 17, and 21 days after tumor inoculation. Non-tumor-bearing, normal C57BL/6 mice of the same age and sex served as controls. Two mice from each group were bled from the retro-orbital sinus, killed, and their axillary, cervical, inguinal, and mesenteric lymph nodes were excised aseptically. The nodes were minced, suspended in cold HBSS, and then cell suspensions were produced by passing the fragments through progressively higher gauge needles attached to a 6-ml syringe. The final lymphocyte concentration was adjusted to  $50 \times 10^6$  viable cells/ml.

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<sup>3</sup> We thank Miss K. Gollahon and Mrs. L. Wickens for expert technical assistance and Mrs. K. Phipps for secretarial assistance.



TABLE 1.—Alloimmune reactivity of C57BL/6 mice to Sa-I

Test conditions	Days post grafting	Corrected percent cytotoxicity*			
		<sup>99m</sup> Tc	<sup>3</sup> H-TDR	<sup>125</sup> I-IUDR	Visual
Test lymphocytes	10	58.0	60.1	63.9	-0.5
	17	32.4	-17.4	32.2	5.1
Test lymphocytes + test sera	10	35.2	57.3	31.6	25.1
	17	19.4	8.1	19.1	16.5
Test lymphocytes + control sera	10	42.7	18.4	40.2	
	17	59.0	-17.3	33.9	
Test sera + complement	10	5.2	-9.5	27.5	13.6
	17	-6.8	10.6	2.9	46.6

\* Corrected percent cytotoxicity =  $\frac{\text{mean cpm}_{\text{control}} - \text{mean cpm}_{\text{test}}}{\text{mean cpm}_{10,000 \text{ Sa-I alone}}}$ . Reactivity of test mice after subcutaneous inoculation of  $20 \times 10^6$  Sa-I cells was determined by the <sup>99m</sup>Tc, <sup>3</sup>H-TDR, and <sup>125</sup>I-IUDR isotopic assays and by visual enumeration of surviving cells.

Control or test lymphocytes ( $5 \times 10^5$  cells) alone or combined with 10  $\mu$ l of control or test sera were added to 6 or 8 replicate wells. Antibody-mediated, complement-dependent cytotoxicity was studied by the addition of 10  $\mu$ l of serum together with an equal volume of a 1:4 dilution of pretested, non-cytotoxic rabbit serum as a complement source. Plates were incubated for 40–48 hours at 37°C in a humidified CO<sub>2</sub> (5%) incubator, then dumped to remove the media, washed twice with HBSS to remove dead and injured cells, air-dried, and fixed with a spray adhesive (Aeroplast<sup>R</sup>). The bottoms of the wells were punched out, and radioactivity was immediately determined with a Nuclear-Chicago Model-1185 gamma counter.

Maximum release of <sup>99m</sup>Tc was produced by subjecting triplicate samples of 10,000 labeled cells, suspended in 1.0 ml distilled water, to 3 cycles of freezing and thawing. After the samples were centrifuged at  $800 \times g$  for 10 minutes, 0.5 cc of supernatant was removed and radioactivity determined. The supernatant contained <9% of the total bound isotope. There was a linear relationship between radioactivity expressed as counts per minute (cpm) and graded numbers of Sa-I cells. Although the actual number of surviving cells per test well could be determined by interpolation, it was more convenient to express the data as corrected percent cytotoxicity.

RESULTS AND DISCUSSION

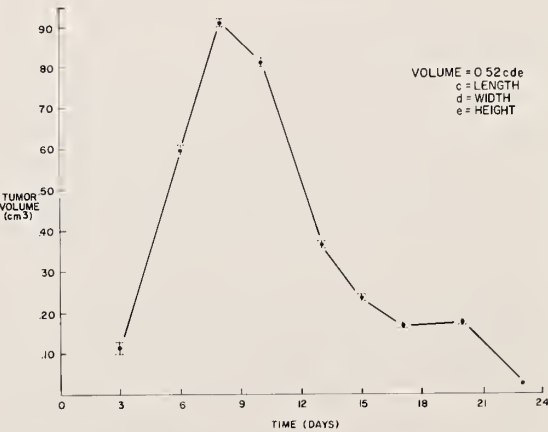
The growth of Sa-I in C57BL/6 mice is presented graphically in text-figure 1. A rapid, linear increase in tumor volume was noted until day 8, at which

time maximum size was attained. Regression was first seen on day 10 and was complete by day 23.

The development and subsequent decline of cell-mediated alloimmune reactivity in C57BL/6 mice inoculated with Sa-I is presented graphically in text-figure 2. The data have been plotted as corrected percent cytotoxicity (C.P.C.), defined as the mean cpm of a control group minus the mean cpm of its respective test group divided by the mean cpm of 10,000 labeled Sa-I target cells alone

$$\left( \text{C.P.C.} = \frac{\overline{\text{cpm}}_{\text{control}} - \overline{\text{cpm}}_{\text{test}}}{\overline{\text{cpm}}_{\text{Sa-I alone}}} \right)$$

The development of immunity paralleled the growth and rejection of the tumor but with about a 2-day delay. Lymphocyte-mediated cytotoxicity

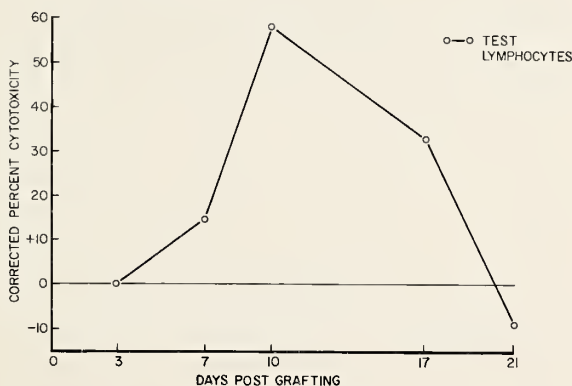


TEXT-FIGURE 1.—Growth of Sa-I in C57BL/6 mice inoculated subcutaneously with  $20 \times 10^6$  Sa-I cells on day 0. Tumor volumes were calculated from the formula  $V = 0.52 \text{ cde}$  as described by Phillips and Gazet (14).

reached a peak on day 10 and declined to the pre-sensitization level by day 21. Antibody-mediated, complement-dependent cytotoxicity was not demonstrable at any time, but this is not particularly surprising, since sarcomas are known to be resistant to such injury (17, 18).

The  $^{99m}\text{Tc}$  microassay has been compared with the  $^3\text{H}$ -TDR and  $^{125}\text{I}$ -IUDR isotopic assays and to a microcytotoxicity assay based on the visual enumeration of surviving cells (table 1). In all experiments, only the  $^{125}\text{I}$ -IUDR assay approximated the sensitivity and reproducibility of the  $^{99m}\text{Tc}$  microassay. Visual enumeration of surviving cells appeared to be the least reproducible method in our hands, most probably due to the removal of target cells and lymphocytes during vigorous washing with HBSS. In contrast, since admixed lymphocytes need not be removed in our assay, only gentle washing with HBSS was required to remove dead and injured target cells.

Since  $^{99m}\text{Tc}$  is widely used for scanning, it should be readily available in most hospitals where diagnostic radioisotopic scanning is performed. Otherwise the cost of the isotope can be expensive. The  $^{99m}\text{Tc}$  microcytotoxicity assay appears to be a sensitive, easily performed test for assessing cell-mediated immunity *in vitro*. The high affinity and short labeling time of the nuclide, together with the fact that it does not appear to be reutilized during the course of an experiment, provide significant advantages over other radioisotopic assays previously described.



TEXT-FIGURE 2.—Development of cell-mediated immunity to Sa-I in C57BL/6 mice. Data have been plotted as corrected percent cytotoxicity, defined as the cpm of a control group minus the mean cpm of its respective test group divided by the mean cpm of 10,000 labeled Sa-I target cells alone.

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## GENERAL DISCUSSION—PART 2

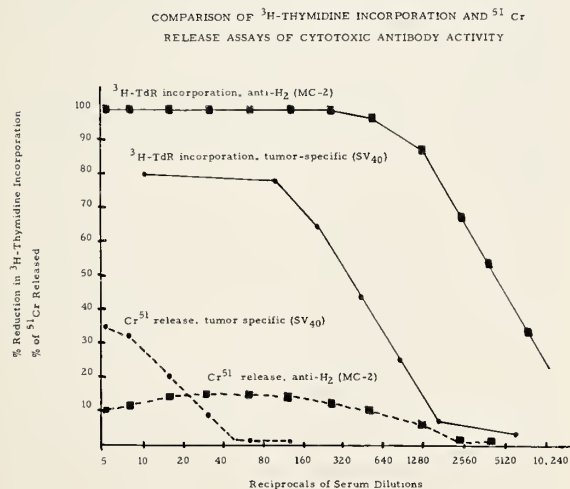
**Rapp:** Thank you, Dr. Witz and Dr. Barth. Dr. McKhann would like to show us 3 figures.

**C. McKhann:** I would like to follow up the very fine remarks of Dr. Levy and point out that some cells in culture are extremely resistant to the effect of antibody in complement. In Dr. Levy's studies, various permutations and combinations of the complements used did *not* necessarily make the cell sensitive to cytotoxic antibody.

Mr. P. Cleveland, a graduate student in our laboratory, began to study this. He looked for more subtle effects of antibody and complement on tumor cells than outright destruction of the cells as evidenced by chromium release or the uptake of dyes. He did this by measuring the incorporation of  $^3\text{H}$ -thymidine into tumor cells growing in culture in the presence of antibody and complement for 24 hours.

Text-figure 1 depicts quite clearly the problem of conventional cytotoxic antibody and the usual methods of measuring it (bottom 2 curves), compared to the effects of antibody and complement on the capacity of the same 2 sarcomas to incorporate tritiated thymidine in culture. One is a murine sarcoma induced by 3-methylcholanthrene (MCA) with H-2 alloantibody; the other is a hamster sarcoma induced by simian virus 40 (SV40) with syngeneic tumor-specific antibody. Titration of the antisera showed significant activity at high dilutions when measured by inhibition of  $^3\text{H}$ -thymidine uptake over 24 hours, but little cytotoxic activity by chromium release.

Text-figure 2 shows an MCA-induced sarcoma of a mouse with syngeneic, tumor-specific antibody recovered at differ-



**TEXT-FIGURE 1.**—Cytotoxic antibody was measured against 2 sarcomas by conventional 1-hour chromium-51 release and by 24-hour inhibition of tritiated thymidine uptake. MC-2 as a murine sarcoma in the antiserum was prepared in allogeneic mice. SV40 is a hamster sarcoma, and the antiserum was prepared in syngeneic hamsters.

ent times and after different approaches to immunization. Again, this demonstrates the capacity of antibody to inhibit the utilization of tritiated thymidine in culture.

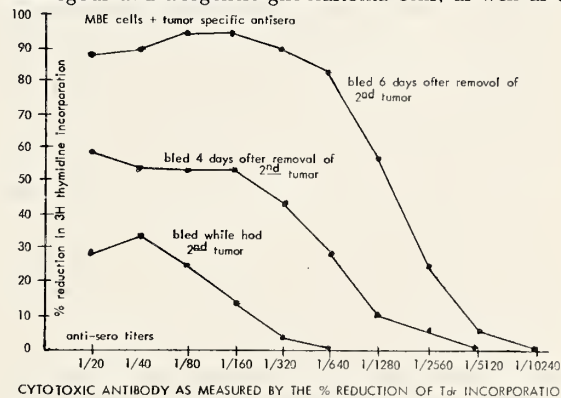
Text-figure 3 is a single experiment showing the capacity of tumor cells to absorb out this specific antibody. The antibody was absorbed once with 16 million cells of the same tumor.

Studies using this technique are still preliminary. It has not been effective with all the tumors that we have tested so far, nor has it worked with all antibody preparations that we've used, even with the same tumor. This last finding appears to have some relationship to the methods at immunization and particularly to the time of collecting the antibody itself. This is not a new concept for measuring cytotoxic effects of antibody and complement on tumor cells, but it does seem to work with some cells that otherwise do not undergo more general, severe, cytotoxic reactions.

**Rapp:** Thank you, Dr. McKhann. Are there now some specific questions for the presenters?

**M. M. Sigel:** Dr. Levy left me in suspense when he talked about some indications for separating antigens involved in the stimulation of lymphocytes and those involved in blocking effects. Would you care to elaborate on these rather interesting remarks?

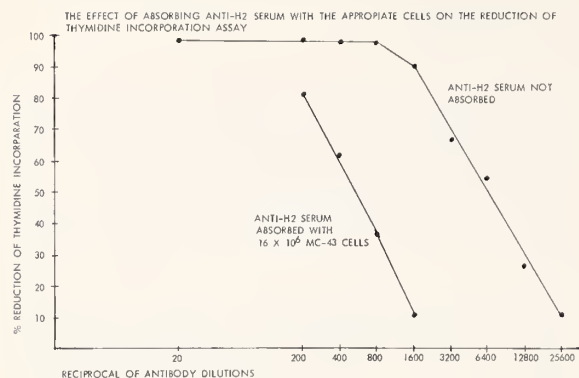
**Levy:** I presented the data at the last Federation Proceedings. Let us consider two groups of patients: those with the histologically anaplastic glioblastoma multiforme and those with the histologically well-differentiated related tumor, the fibrillary astrocytoma. Cell-mediated and blocking activity was assessed in these patients, by use of our modified Hellström procedure<sup>1</sup> against glioblastoma, astrocytoma, melanoma, and other tumor target cells. Lymphocytes from glioblastoma patients are cytotoxic to both autologous and allogeneic glioblastoma cells, as well as to



**TEXT-FIGURE 2.**—The cytotoxic effects of syngeneic tumor-specific antisera against an MCA sarcoma measured by inhibition of incorporation of tritiated thymidine over 24 hours.

<sup>1</sup> LEVY NL, MAHALEY MS JR, DAY ED: In vitro demonstration of cell-mediated immunity to human brain tumors. *Cancer Res* 32:477-482, 1972.





TEXT-FIGURE 3.—Effect of specific absorption of cytotoxic antisera as measured by inhibition of incorporation of tritiated thymidine.

astrocytoma and melanoma cells. This is not a nonspecific effect, since these lymphocytes are not cytotoxic to normal glial cells or fibroblasts autologous to the tumor cells or to sarcoma, colon carcinoma, or breast carcinoma cells. Lymphocytes from fibrillary astrocytoma patients are cytotoxic to both glioblastoma and astrocytoma cells but not to melanoma cells. This is one unusual cross-reactivity pattern, but the more fascinating one arises from our studies with blocking sera. Blocking sera from glioblastoma patients will abrogate the cytotoxic activity against glioblastoma cells, be it mediated by lymphs from glioblastoma, astrocytoma, or melanoma patients, but will not block the cytotoxicity of these same lymphs against astrocytoma or melanoma target cells. Similarly, blocking serum from astrocytoma patients will block the cell-mediated destruction only of astrocytoma target cells, though lymphs from the blocking serum donor are cytotoxic to glioblastoma target cells as well. We have tried a wide range of blocking serum dilutions from 1:3 to 1:100 and find the patterns to persist.

Absorption studies, both with the serum and with the immune lymphocytes, the latter in collaboration with Dr. Gideon Berke, are in progress. If these patterns persist in the face of absorption experiments, they will strongly suggest that different determinants or different conformational forms of the same determinant are involved in blocking as in cell-mediated reactivity. This concept is certainly not without precedent if we consider the hapten-carrier story, in which the cell-mediated immune component is directed against the large carrier molecule and the humoral component against the smaller haptenic determinant.

**M. M. Black:** I should like to add to the complexity that the previous speakers have mentioned in regard to *in vitro* and *in vivo* tests of the biologic system. Even though an attempt was made to correlate a particular test with the growth of the tumor, in the human situation the local growth is often inconsequential. We don't care how big a breast tumor grows locally, *e.g.*, cytosarcoma phylloids. Simple removal ends the problem. Clinically the problem in cancer is the dissemination and the infiltration, which may have very little correlation to the local growth.

We are all familiar with occult carcinomas where the

primary tumor is almost microscopic, and yet the patient is dying of dissemination.

And to extend what Dr. Witz said, this should be strongly emphasized. We think of a patient as having a particular level of immunologic response; since we now have on our immunologic glasses, we think that everything that happens is therefore immunologic and constant throughout the body. This is far from true, as any clinician knows. He may have one focus of metastasis that grows, another one that's doing nothing, and another one that's regressing. This may also be seen in regard to the effects of any chemotherapeutic agent. So I hope we don't oversimplify the problem as it exists in man.

**J. Stjernswärd:** This discussion is still missing a unifying concept. Earlier speakers (especially 2 speakers) have clearly shown how difficult it is to find a relevant test of really what is happening *in vivo*. First they demonstrated all the pitfalls. Then when they demonstrated their own experiments, they fell in the trap: When have you seen a tumor arising in a Millipore chamber *in vivo* in a cancer patient?

The other concept is the skin graft as a model which definitely is not comparable with a single tumor cell. As has been pointed out by 2 speakers earlier, the rejection mechanism of the skin graft is different by this model, because it's probably—or it cannot be excluded—that it is depending on reactions that occur in the capillaries supplying the graft.

There is another point that I would like elucidated. In all the tests that have been discussed, one point has been missing: Everyone works with his target cells and thinks these cells are representative for the *in vivo* situation; but if we have, as Dr. Witz pointed out, blocking antibodies on the tumor cells, we have to look at the tumor cell itself.

These are just some of the problems we have ignored in our present analysis. As soon as we take out the tumor cell and make a tissue culture for some days, we have an unknown selection of cells. We don't even know if they are tumor cells. We may have antigenic loss or gain or depression.

If possible, could one of the Chairmen give a synthesis: Where should the poor experimentalist start? Should he continue with his experimental artifact, or where should he go?

**Bartlett:** Possibly we have the same problem here that the physical scientists express by the uncertainty principle: Whenever we study one component of a system, we probably alter the system. The goal of having a model system that duplicates exactly what happens in the undisturbed primary host may be unrealistic. There are several approaches to this problem. The first is to critically assess the model systems we are using. This can be done, theoretically, in part by direct comparison of several techniques and in part by application of the same technique to different problems and materials. A second approach is to develop new models which, though not *exact* replicas of the *in situ* primary tumor, may permit us to look at different aspects of the problem and which may complement the limitation of other models.

**LoBuglio:** I wish to comment on the need for cultured cell lines and the limitations in interpretation with cultured cell lines: It is possible to demonstrate MIF release in

human systems to noncultured cell preparations; *i.e.*, one can take tumor tissue and, by using the cell preparation as it exists (either intact or lysed), can demonstrate specific MIF release by the patients' lymphocytes. Therefore, this at least demonstrates the fact that there is cellular immunity to the tumor as it exists. It also points out the fact that the difficulty in doing cytotoxicity tests in this way is interesting, because it may well be interpreted that the  $\gamma$ -globulin coating of tumor cells *in vivo* prevents a direct demonstration of cytotoxicity, but may not prevent demonstration of immunity by MIF release. So, at least in certain instances, you can detect tumor immunity without having cultured cell lines for your antigen source.

**S. Deodhar:** With respect to Dr. Black's comments about dissemination of tumor cells, I would like to mention one *in vivo* technique we have used extensively in our laboratory. It has to do with estimating metastases from tumors implanted in the feet of mice.

What we found essentially is that, in mice suppressed immunologically by various agents, the degree of metastases to the region of popliteal nodes is markedly higher than in normal mice with intact immune response, or in mice that have been treated with irradiated tumor cells to enhance their immune response. This particular system admittedly is at best semiquantitative, but its correlation with respect to *in vitro* techniques has been excellent.

**C. C Congdon:** Mr. Chairman, there was a suggestion at the start of this meeting that a goal might be to arrive at a consensus in reference to the three challenges of Frank Rauscher. I wonder, are we getting anywhere with a consensus on these techniques and methods?

**Rapp:** Does someone want to comment specifically on that?

Obviously the best solution to this problem would be to prevent the disease. I should like to remind you that this is supposed to be a conference on immunology of carcinogenesis, and so far we have been talking about tumor cells!

Clearly, if we could demonstrate etiologic agents causing these diseases, it would raise the possibility of producing vaccines and thus prevention. As you know, there is some hope along these lines—at least with a chicken lymphoma, Marek's disease caused by a herpesvirus. By vaccinating these chickens with similar virus grown in turkeys, this disease can be prevented.

What about the possibility that we can study chemical, nonviral carcinogens themselves as immunogens? We are beginning some work along these lines. Dr. Heise, would you comment on some of the preliminary work you are doing in this area?

**E. R. Heise:** The question is whether carcinogen-protein conjugates, which can be synthesized in the laboratory, could be used in any way to inhibit the development of a chemically induced tumor. We are not doing that experiment at this time. I think Dr. Pomeranz will be performing such experiments. Our own work is concerned with the allergenic properties exhibited by many of the chemical carcinogens.

But the reagents I believe can be synthesized for the detection, by *in vitro* methods, of cell-mediated sensitivity to chemical carcinogens.

The major difficulty is that we do not know the nature of

the protein in the body to which the chemical carcinogen interacts to form the complete antigen. Presumably often this is a skin protein. We have isolated a proteinaceous material through the use of 3 M KCl that will conjugate to the isocyanate derivative of benzpyrene.

A lot of the data does indicate that the carrier substance is in the site of the application of the chemical carcinogen. Experiments have shown that excision of the site of hapten application up to several days of postsensitization will markedly interfere with the induction of contact sensitivity.

Possibly, though, serum proteins or substances in the lymph are involved as carriers.

**Rapp:** I think a partial answer to some of these problems is that we've got to develop those models that are already available and get new ones that can be described as primary autochthonous models.

**A. J. Girardi:** You brought up one situation in which you have protection against a virus-induced tumor. It's an extremely effective vaccine used in the Marek's disease.

Now not to complicate the picture at all but to really point to the complex nature: What is Marek's disease vaccine doing? When you immunize with it, it's not preventing infection by the agent, the growth of the agent, or even some of the mild proliferation of tissue infected by the agent. And yet the bird survives!

Even at this level, after curing a situation clinically, the mechanism isn't really known. But what is really going on in Marek's disease may be very important for the control of other infections.

**Rapp:** That indicates, as Dr. Borsos said earlier, that the endpoint has to be cancer control: All the other things are just ancillary.

**Sigel:** I would like to reply to the questions raised by previous commentators regarding the validity of the various *in vitro* procedures in relation to the *in vivo* process. It seems to me that, in addition to trying different models and different animal species, what is really needed is the sort of study that Dr. Black will tell us about later—perhaps where one compares the same type of tumor in man in relation to its aggressiveness or *in situ* situation. This is a good beginning to try to pin down the validity of the various serologic and lymphocytic reactions in the context of the cancer itself. How do the components of immunity behave in the *in situ* restraint situation, in contrast with what they do or do not do when the same cancer becomes highly aggressive?

**C. E. Mawas:** One short comment: When, in an allogeneic system, one measures in parallel the cell-mediated immunity (CMI) response against a tumor cell and the humoral response (serum antibody titers as well as complement-dependent cellular cytotoxicity), a certain number of observations can be made: 1) There is a strain variation for the ability to develop a good CMI (C3H/HeHa > DBA/2/J when given injections of EL 4 cells from a C57BL/6 tumor; inability of C3H/HeHa to develop a good CMI against L1210); 2) the humoral response is also strain dependent and not always directed against the same antigen(s) as the CMI is; 3) only the animals developing both responses can reject a load of  $3 \times 10^7$  EL 4 tumor cells: DBA/2 are killed by the tumor despite the cytotoxic serum in these animals; and 4) obviously *in vitro* cytotoxicity in the presence of xenogeneic C has no obvious parallel with *in vivo* results.

**Rapp:** This gets us farther away from an answer to the problem. It's time we stopped doing a lot of allogeneic experiments and began experimenting with the primary autochthonous host. Even the transplant has its problems.

**J. W. Thomas:** Dr. Bartlett, do you have any data on the tumor cells frozen by such a technique as DSMO and later used in conjunction with bacille Calmette-Guérin (BCG) in your immunization?

**Bartlett:** No!

**Rapp:** Any other questions?

**R. B. Herberman:** One major issue I would like to bring up about the studies of Dr. Witz is that showing immunoglobulins bound to tumor cells and being able to elute them off are not really the same as showing specific antibodies bound to the cell. Does Dr. Witz have information as to whether the immunoglobulin he eluted off had antibody activity?

**I. P. Witz:** I fully agree, but because of time limitation I just wanted to emphasize the importance of looking at the humoral immune components at the tumor site. I, as well as others, have some indications that eluted immunoglobulins have antibody activity. One indication is that the eluted antibody could enhance tumor growth *in vivo*.

**Levy:** Any studies done on antisera eluted at low pH must be interpreted with caution in light of the classic experiments which showed irreversible denaturation of the Fc fragment at pH <4. Such effects would not alter the antibody's specificity but might alter its biologic activity. For instance, so-called blocking factors isolated by low pH elution from tumor cells could be cytotoxic antibodies whose Fc fragment has been altered. Residual Fc-related

function of the antibody still does not rule out such effects, moreover.

**H. T. Wepsic:** One way I sometimes think about the immune response in relationship to animal models is that they enable us to look at how the host response fails. The host response seems to be present in many tumors. Indeed our sophistication in showing this has become better day by day, but the question I have is just how does the response fail? There is enough literature now to show perhaps 6 or 7 different ways in which tumors have been shown to grow progressively in different systems. Should we be looking at the human material in this way? Instead of showing that the patient has an immune response to the tumor, we should look at why the response against the tumor fails to prevent the metastases and inevitable death of the host.

**Rapp:** Do you want to comment on that, Dr. Witz?

**Witz:** Concerning Dr. Levy's comment, Dr. DeVaux St. Cyr in Paris has eluted gamma 2 from SV40-induced hamster tumors at pH around 3.0. The eluted gamma 2 was still cytotoxic. Your suggestions that the Fc fragment is denatured at a pH <4 seem not to be always correct.

**J. M. Mason:** I have been working with mouse mammary tumor systems with regard to eluting antibodies. We have been using 0.2% sodium deoxycholate from primary tumors. We not only get tumor antigen out but also are eluting antibodies using the detergent. These antibodies, or a fraction of them, have an altered electrophoretic mobility, suggesting that the complexes that you mentioned earlier, Dr. Witz, may indeed be present.

**Rapp:** I think that the speakers deserve a round of applause.



## **SESSION 2**

### **Antigens in Preneoplastic Tissue During Tumorigenesis**

**Chairmen: M. A. Lappé and David W. Weiss**





# Possible Significance of Immune Recognition of Preneoplastic and Neoplastic Cell Surfaces<sup>1</sup>

M. A. Lappé, *Institute of Society, Ethics and the Life Sciences, Hastings-on-Hudson, New York 10706*

**SUMMARY**—The paradoxical existence of both adaptive and maladaptive immune responses to neoplastic cells suggested that selective forces other than those for protection against tumors of late onset shaped the evolution of a cell-surface recognition system. Adaptive immune responses (immune surveillance) were reviewed and found wanting. Immune surveillance was deficient and ineffective in most tumor systems, allowing the persistence of tumor cell foci after initiation and incompletely eliminating tumor cells after apparent immune-mediated regression. Maladaptive responses (immune stimulation) may have participated in the emergence of antigenic tumor types and reinforced their growth. A common explanation for the operation of both of these immune functions could be found in maternal-fetal interactions and in the need for prenatal and postnatal surveillance against cell lines which fail to complete specific developmental sequences. Maternal-fetal interactions, in which histocompatibility favors the fetus, were reviewed; the apparent successful immune control of undifferentiated tumor types, which may have originated from blocks in differentiation, is offered as evidence. It is hypothesized that a key function of the immune system is to regulate the expression of components on the cell surface and thereby maintain cell types at an appropriate level of differentiation.—*Natl Cancer Inst Monogr* 35: 49–55, 1972.

A WIDE variety of cell-surface changes accompany the transition from normal to neoplastic in virtually all tissue types characteristic of higher organisms. Some of these changes, *e.g.*, the observed increase in agglutinability of tumor cells by plant lectins, were previously thought to reflect steric alterations in surface carbohydrate biochemistry that were simply incidental to the neoplastic transformation, now appear to be directly related to the property of malignancy (1). Other changes, like the increase in synthesis of cell-coat material, may reflect a more basic derangement in mechanisms in some tumor cells. Nevertheless they do not indicate malignancy in all neoplastic cell types (2). However, cell-surface changes detected immuno-

logically *i.e.*, those associated with the appearance of neoantigens, fetoantigens, or tumor-specific antigens, are of greater interest because their occurrence affords the immunologically competent host the prospect of immunologic interaction with the neoplastic tissue. This interaction may be, in the host's reference frame, adaptive, neutral, or maladaptive. For this discussion, I will focus primarily on the 2 forms of host response that have generated the greatest controversy: those appearing to be adaptive (immune surveillance) and those appearing to be maladaptive (immune stimulation).

## Adaptive Host Immune Responses to Neoplasia

Those immune responses which could hypothetically suppress or eliminate tumor cell growth have attracted great attention and interest in recent

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<sup>1</sup> Presented at Conference on Immunology of Carcinogenesis, held by the Oak Ridge National Laboratory at Gatlinburg, Tenn., May 8–11, 1972.

years. The phrase "immunologic surveillance," first coined in 1959 by Thomas (3), proved to be a convenient means of anticipating an adaptive tumor-limiting function of the immune system, but unfortunately it contains an evolutionary pejorative implying that immune systems evolved to "seek out and destroy" nests of tumor cells wherever and whenever they might occur. Such a usage, reinforced over the years by Burnet (4-8), casts the immune system in a role difficult to reconcile with the selective forces operating on higher organisms. Selective pressures operate almost exclusively before and during the fertile period of life, whereas most (but not all) tumors occur with exponentially increasing frequency *after* the reproductive years. For the late-appearing class of tumors, immune mechanisms appear to exert only a holding action, delaying but not countering tumor appearance until after the onset of senescence (9-11). This incomplete operation of immune surveillance may reflect the vestiges of a much more efficient system normally operating during the late embryonic and immediate postnatal period which suppresses or forces the differentiation of cell types which still retain embryonic surface characteristics.

### General Failure of Immune Surveillance

The varieties of adaptive immune response generally condensed under the heading "immune surveillance" are probably much broader than previously recognized. In addition to the well-known activity of thymus-dependent lymphocytes which participate in the cell-mediated arm of the immune response, a number of soluble lymphocyte mediators may be expected to become increasingly recognized as participants in the destruction of tumors by immunologic means. In a recent summary, Mackler (12) identified at least 4 mediators that probably participate in the immune reaction against neoplastic growths: 1) mitogenic factor, which recruits previously uncommitted lymphocytes; 2) migration-inhibition factor (MIF) which holds phagocytic cells in the inflamed area; 3) lymphotoxin, which participates in the destruction of tumor cell membranes; and 4) transfer factor, which recruits specifically sensitized lymphocytes.

Although it is becoming increasingly apparent that traditional mechanisms of immune surveillance (*i.e.*, cell-mediated responses) can and do fail, it is tempting to speculate that inadequate

responses involving soluble factors constitute a previously unrecognized source of surveillance failure. For example, blocking factors in the serum probably not only interfere in general with cell-mediated tumor immunity (13) but also may specifically prevent the MIF from eliciting the accumulations of macrophages normally seen during some forms of tumor growth (14). Inadequate concentrations of lymphotoxin may also be responsible for failure to kill target cells, and at low dosages, lymphotoxin may even stimulate cellular DNA synthesis (15).

### Evidence for Incomplete Immune Elimination of Tumors in Adult Animals

The general pattern of evidence for increased tumor incidence after immune suppression in experimental animals is compatible with a hypothesized "holding action" of the immune system. The overwhelming impression is that immunity increases tumor latency but does *not* really decrease tumor number [see review by Lappé (11)]. Even in man, where immune suppression is accompanied by the appearance of new nonlymphoid neoplasms (16), the interval between suppression and clinically apparent neoplasia is sufficiently short to suggest that tumor cell populations were already at more than the one-cell stage. This type of study gives the observer the impression that neoplastic cell masses are simply held in a resting stage before release. DeCosse and Gelfant (17) offered experimental evidence for such a model of immune suppression.

Other explanations for surveillance failure are possible. Rowland *et al.* (18) demonstrated that tumor progression, but not growth *per se* [cf (19)], was accompanied by a specific failure of competent cells to elaborate specific antibody. This effect appeared related to the presence of residual tumor cells (20) and could be duplicated experimentally by the injection of tumor cells (20, 21) but not antibody (22). Thus "enhancement" *per se* is an incomplete explanation of surveillance failure [cf. (23)].

The immune system also appears to be only partially effective in eliminating tumors through regression. When tumors have undergone a putatively immunologic regression, they may be retrieved if cell proliferation is provoked by an appropriate stimulus. For example, MacKenzie and Rous (24) found, in their classic experiments in rabbits,



that "disking" the site of a former papilloma that had disappeared on a rabbit ear (*i.e.*, punching a hole with one edge cutting through the former papilloma site) caused the presumptively regressed tumor to appear. In my studies (25), stimulation of precisely plotted papilloma-regression sites with croton oil produced a small (4/30) but dramatic recrudescence of the same papillomas that had regressed up to 120 days previously. These observations, coupled with the well-known fact of the irreversibility of initiation, strongly suggest that immunologic surveillance only rarely permanently destroys *in situ* tumor-cell populations. The apparent success in demonstrating surveillance in some systems (26) can be attributed either to highly antigenic tumor-cell populations or to an adjuvant-type effect on the host's immune system of the mode of tumor presentation (11). Demonstrations of surveillance against spontaneous tumors was the exception (27), not the rule.

One explanation for surveillance failure is the relative inaccessibility of *in situ* tumor cells. In a systematic attempt to demonstrate a reduced incidence of *in situ* papilloma with time (table 1), I found no significant difference in the number of papillomas that could be retrieved by graft promotion 1, 2, 6, or 9 months after initiation in immunologically competent hosts.

This kind of result in a non-carcinogen-depressed initiation-promotion system strongly reinforces the original observation of Berenblum and Shubik (28) that initiation is virtually irreversible. Although I have proffered alternative explanations (25, 26), contemporary data [*cf.* (11)] indicate that

immunologic surveillance does not operate during the latent period of tumorigenesis.

### Maladaptive Host Immune Responses to Neoplasia

Prehn and I have suggested that certain evolutionary forces have shaped tissue responses to immunologic recognition so that *stimulation* rather than destruction of cells may be beneficial (29). The evidence supporting this contention comes primarily from embryologic studies which demonstrate a selective advantage for the antigenically dissimilar fetus [*cf.* (30)]. That this phenomenon could also be operative for antigenic tumor cells has now been experimentally verified by Prehn (31).

With tumors, the operation of immune stimulation affords a novel (albeit partial) explanation for both the general failure of immune surveillance and the success of small foci of demonstrably antigenic cells to grow out in the face of an evident immune response [*reviewed in* (29)]. The observation that small doses of tumor cells can "sneak through" and escape immune surveillance has been ascribed to a delay in host immunization, giving the tumor focus a "headstart" on the host immune response (32). As Prehn and I pointed out (29), "sneaking through" not only occurs in the presence of an immune response but also appears to occur more readily when implants are placed in a previously immunized host (33).

The fact that substances which generally stimulate the immune response can to a limited extent curtail tumor development (34-36) demonstrates that immune surveillance is not completely ineffective. However, the observation that immune stimulation with bacillus Calmette-Guérin (BCG) or a methanol extraction residue of BCG can sometimes stimulate tumor growth (37-39) reinforces the possibility that immune stimulation comprises a significant component of maladaptive immune responses. Whether this apparent stimulation of growth is due to classical "tumor enhancement" or reflects the operation of immune stimulation remains to be demonstrated.

Since, under experimental conditions, an immune reaction can encourage tumor growth (31), possibly immune stimulation can explain both the widespread occurrence of tumor-specific antigens and the appearance of like antigens in other than the original tumor cell population. The first por-

TABLE 1.—Absence of *in situ* surveillance in the autochthonous host (BALB/cAnN1er females); initiation involved 10 days' exposure to a disc containing 10% 3-methylcholanthrene; grafting was to immunologically anergic hosts\*

Days after initiation	Number papillomas†	
	Number grafts	Mean $\pm$ SE‡
30	12/42	0.29 $\pm$ 0.07
60	11/53	.21 $\pm$ .06
180	4/18	.22 $\pm$ .10
260	4/24	.17 $\pm$ .08

\* Based on unpublished data from (25).

† Papilloma populations were assayed by grafting the initiated skin to immunologically unreactive isologous hosts (thymectomized as adults and irradiated with 450 R) to minimize any immune surveillance in the graft recipients.

‡ SE = standard error of the mean.



tion of this concept, a kind of immunoselection in reverse, was initially suggested by Prehn and me (29). The second portion would be more tenable if immune factors were implicated directly in cell-surface changes. The possible role of immune mediators in repressing cell-surface changes in tumors is implicit in the work which first demonstrated the lability of the TL antigen (40). In this system, the presence or absence of antibody appeared to repress the expression of TL antigen in leukemic cells (41). Other studies have now indicated that antibody-mediated, cell-surface antigen repression may be a general phenomenon (42).

To fit the hypothesis that immune stimulation selects for common antigens, however, some component of the immune response would have to be shown to *de-repress* antigens on the cell surface. There is at least one, albeit nonmammalian, model for cell-surface antigen induction by antibody which fits a *de-repression* model. In bacteria, sex pili (and their associated antigen) can be induced by exposure of the cells to low doses of antibody (43). Such a capacity would be evolutionarily advantageous for bacteria exposed to incipient immune responses. Conceivably, such cell-surface responsivity to antibody provides a new model for the reconsideration of traditional theories of the participation of lymphoid tissues in growth control in general (44).

The ability of immune stimulation to reinforce tumor growth, at least at initial low levels of immune reactivity (37), provides an explanation for the proliferation of tumor antigenic specificities. An evolutionary model for the emergence of tumor antigenic diversity can be found in host-parasite models. The work of Gemmell (45) suggests how selective pressures for both antigenic diversity and antigenic cross-reactivity could have evolved in tapeworms (45). There are at least 2 classes of antigenic configurations in tapeworms, one of which is species-specific. This antigen provokes an initial host response which prevents the establishment of any subsequent challenge infection. The initial colonization of the host by the *first* tenia is often successful even in the face of an immune response. Consequently, an early immune reaction generated by the first infecting organisms may encourage already established taeniae, while reducing the prospect of superinfection (and concomitant host disability) by like antigen-bearing members of the same species. By analogy then, conceivably the

well-known antigenic diversity in tumors is stimulated only after an immune response against the original specificity has exerted its selective pressure (29). Only after a certain level of immune responsiveness has been reached would the reaction begin to select *against* like antigenic tumors. Such a hypothesis could be tested by maintaining the immune response to a specific tumor-specific antigen at a controlled level during carcinogenesis. Predictably, the first wave of tumors would then be expected to express the same or cross-reacting antigenicity. In fact, all chemically induced tumors, while individually specific, appear to contain an overriding common antigenicity (46-50). Where common antigenicity has been conclusively excluded, the model system entailed *in vitro* or diffusion chamber exposure to carcinogen (51) in which selection through immune stimulation for common types would have been excluded.

### Synthesis

It is almost axiomatic that, if maladaptive responses operate in a physiologic system, the adaptive functions of the same system must (in an evolutionary sense) far outweigh the maladaptive ones. It is tempting to speculate that the primary function of a system which recognizes cell-surface changes is to confer a selective advantage to the host during pregnancy (52). There is growing evidence that the immune system is intimately involved in the operation of normal reproductive processes (53). Indeed, the fact that a preponderance of immunologic reactivity is directed toward histocompatibility antigens points to a relatively unappreciated intraspecific value of immunologic recognition (54). If the immune system has a crucial function in establishing, maintaining, or modulating the differentiation of the growing embryo, then the same system might continue to perform related functions in the adult. The apparent success of surveillance in suppressing and modulating cell phenotypes, rather than *eliminating* the same cells, supports this contention.

Schalk and I previously reviewed the evidence for the operation of immune mechanisms for potentiating the early phases of pregnancy (52). I present here the as yet speculative evidence linking the immune system to a regulation of cell-surface changes during embryogenesis. Growing evidence demonstrates the close relationship between em-

byronic and tumor development (55). Most recently, it has proved possible to immunize against simian virus 40 oncogenesis by sensitization with fetal antigen (56). Embryonic development and differentiation *per se* may pose sufficient threats of progressive cellular overgrowth through either failure to complete a differentiation sequence (57) or through *de novo* neoplasia (e.g., trophoblast giving rise to choriocarcinoma), to have exerted selective forces for shaping a powerful immune recognition system. The evidence for the operation of immune factors in determining the course of choriocarcinoma has been well established (58). Both blood-group and histocompatibility genetic loci are important factors in the pathogenesis of this neoplasm. [See the recent letter by Bagshawe and Lawler (59)]. The need for positing a protective function of an immune system during pregnancy is further reinforced by recent studies demonstrating that, under proper hormonal conditions, even xenogenic tumor cells will grow in the mammalian uterus (60).

#### Relation of Immune Response to Embryonic Differentiation

The existence of a class of tumors arising predominantly during the early postnatal period, a growing number of which are demonstrably antigenic [cf. (61)], provides a clue to the origin and function of tumor recognition by the immune system. Selective pressures would be expected to be stronger for host control of these early neoplasms, rather than neoplasms of later onset. The nature of this host control, however, may need only have been akin to embryonic modulation to regulate effectively some forms of tumor differentiation (57).

The preponderant tumor types within the category of tumors whose incidence curves occur before the classic delayed-onset curves are those which appear to have an embryologic origin. Thus there may well be strong evolutionary pressures forcing the fetal (and perhaps maternal) host to participate in the control of incomplete or aberrant differentiation. Seilern-Aspang and Kratochwil (62, 63) presented dramatic evidence for the operation of differentiation-producing mechanisms in tumors induced in the newt. There is a growing body of evidence that, in higher animals, immunity regulates the differentiation of tumors of embryonic origin. Specifically, neuroblastoma, which in man

constitutes the major tumor in this category, is subject to some degree of immune control (61). Strongly suggesting an effective mechanism for host regulation is the observation that neuroblastoma is 40 times more common at autopsy in infants under 3 months old (64) than the incidence expected from overt clinical cases (65). Moreover, the tests demonstrating host immunity also appear to demonstrate *maternal* immune recognition of the tumor, supporting the notion of a general, evolutionarily adaptive function of immune recognition of aberrant embryonic cells.

In at least a few of these tumor types (including neuroblastoma), regression occurs as the result of differentiation. The potential for neuroblastoma differentiation was directly demonstrated following X irradiation (66). A strong possibility exists that differentiation of neoplastic tissues is mediated by immune mechanisms. Artzt and Bennett (57) and Basombrío and Prehn (67) reported immune regression of tumors of embryonic origin. Although interstrain histocompatibility differences possibly contributed to their regression, the general impression is that, in these systems, lymphocytic infiltration contributes to tumor differentiation (68).

In at least one model system (preinvasive breast lesions in the mouse), where the phenomenon of immune-mediated "differentiation" appears to occur, it is now apparent that normal stroma accompanies the hyperplastic nodular tissues. However, the fact that, after immune infiltration and the resumption of the normal differentiated appearance, cells with malignant potential are *still* recoverable (69) supports the contention that a key function of the immune system is to regulate the morphologic expression of "normalcy" [cf. (70)]. Such a conclusion would be consistent with a model of the immune system as primarily a modulator of cell-surface configuration and with the fact that immune systems to date have proved to be minimally effective in eliminating the malignant cells characteristic of late-onset tumorigenesis.

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# Host Response to Premalignant Mammary Tissues<sup>1, 2</sup>

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**SUMMARY**—The biologic characteristics of mammary tissue during the development of malignancy were studied by use of a mammary-gland transplantation technique. Normal mammary gland was composed of at least 3 separate populations of cells representing 3 types: ductal epithelial, alveolar epithelial, and myoepithelial. Serially transplantable outgrowth lines of premalignant tissues involving neoplastic cells of any one of these 3 types were isolated from primary lesions arising spontaneously or induced by 3-methylcholanthrene (MCA) or mammary tumor virus. Sequential progression to malignancy occurred within each line. Neoplastic premalignant cells of each type were frequently associated with normal parenchymal cells of the same and different types. This association was required for the survival of some neoplastic components before, but not after, the stage of malignancy was reached. The neoplastic component of premalignant tissues induced by MCA expressed non-cross-reacting neoantigenicity which persisted unchanged during progression to malignancy. The malignant tissues were, however, more resistant to immune destruction than the premalignant tissues of origin. Transplanted antigenic premalignant lesions made to simulate primary *in situ* lesions failed to immunize the host, but remained antigenic and susceptible to immune destruction.—*Natl Cancer Inst Monogr* 35: 57–71, 1972.

THESE INVESTIGATIONS were initiated in 1962 in the laboratory of Dr. R. T. Prehn, then at the University of Washington, to study the relationship of antigenicity to oncogenesis. A method was sought for the transplantation of premalignant tissues which would be likely to give rise to anti-

genic malignancies. DeOme *et al.* (1), using their mammary-gland clearing and transplantation technique, studied premalignant mammary tissues induced by mammary tumor virus (MTV). The malignant carcinomas derived from these lesions were believed at that time, however, to be nonantigenic. Prehn (2) had shown that malignant mammary tumors induced by 3-methylcholanthrene (MCA) were antigenic. Therefore, I established serially transplantable, premalignant tissues from lesions induced by MCA. Then I studied these tissues by classical immunization and challenge procedures, for their capacity to induce transplantation resistance.

Three different types of neoplastic premalignant tissues exhibited, respectively, alveolar, ductal, and squamous differentiation. Each of these types of premalignant tissues expressed specific neoantigenicity which persisted unchanged during pro-

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<sup>3</sup> These studies were done in the laboratory of Dr. Prehn, without whose support, direction, and continuing patience this work would not have been accomplished.

gression to malignancy. Alveolar and ductal premalignant tissues unexpectedly gave rise to normal outgrowth when subjected to specific immune response, whereas most premalignant squamous lesions and all malignant variants from all types of tissues were completely destroyed when rejected by immunity.

To study the nature and origin of the normal tissues derived from premalignant tissues, I used mammary glands composed of a mosaic of cells of 2 different genotypes. These studies showed that normal mammary glands consist of 3 separate populations of cells representing 3 different types: alveolar epithelial, ductal epithelial, and myoepithelial. The 3 types of neoplasia involve the neoplastic transformation of each of these 3 types of cells. The neoplastic premalignant cells of each type were frequently associated with normal cells, and this association was required for the survival of some neoplastic premalignant but not malignant variant populations.

This relationship of premalignant cells to normal parenchymal and stromal elements helps to explain the differences between the growth and immunizing characteristics and response to immunity of premalignant and malignant tissues.

Some of these studies were summarized previously (3).

## MATERIALS AND METHODS

*Experimental animals.*—Inbred mice were of the following strains: (abbreviations in parentheses) BALB/cAnNIcr (C), DBA/2AnPrIcr (from the Seattle colony of Dr. Prehn transferred in 1966 to ICR) (D2), C3H/HeNIcr (C3H), C3HfB/NIcr (C3Hf), C57BL/6JNIcr (B6); F<sub>1</sub> hybrids between these, and (C × D2) hybrids fostered by C3H mothers and, thus, having the standard C3H form of MTV (MTV-S). These are designated (C × D2)<sup>+</sup> to distinguish them from the regular (C × D2) mice believed to lack active forms of MTV and henceforth designated (C × D2)<sup>-</sup>. (BALB/cAn × C3H/-He)F<sub>1</sub> mice from the stocks of the Seattle laboratory of Dr. Prehn were also used in earlier studies; these are designated (C × C3H)<sup>Sea</sup>.

*Mammary gland clearing and transplantation.*—The mammary-gland transplantation technique of DeOme *et al.* (1) was used. The rudimentary mammary epithelial tree and the adjacent portion of the #4 (inguinal) mammary fat pad were

extirpated in prepubertal females through a short ventral midline skin incision. After this "clearing" operation, the rest of the fat pad remained free of host epithelium and served as a transplantation site for experimental tissues. The experimental tissues grew in their natural environment, and the cleared fat pad thus served essentially as an *in vivo* organ culture chamber. The experimental tissues could be readily identified and transplanted serially in histocompatible recipients.

*Induction, isolation, and serial transplantation of premalignant tissues.*—Serially transplantable lines of hyperplastic, premalignant tissues were established from transplants of hyperplastic mammary lesions. Hyperplastic lesions were obtained from mice with MTV or from mice after MCA administration with or without MTV or the hormonal stimulation of pregnancy. MCA, 1 mg in 0.2 cc olive oil, was administered intragastrically in 6–17 doses at intervals of from 2–7 days. Primary transplants of hyperplastic tissues frequently gave rise to mixed growths of hyperplastic and normal tissues. Selection of the hyperplastic portions of these outgrowths for serial transplantation resulted in the isolation of cellular populations which routinely produced morphologically homogeneous initial hyperplastic outgrowths (fig. 1). After several weeks or months, many malignant variants developed in these outgrowths (fig. 1). If these variant areas were avoided in the selection of tissues for serial transplantation, the original, or precursor, forms of the premalignant tissue could be maintained as established outgrowth lines in serial transplantation through many generations covering several years.

*Immunization and challenge.*—In experiments designed to test the immunizing capacity and response to specific immunity of premalignant or malignant mammary tissues in syngeneic animals, experimental and control groups of female mice with host-gland-free mammary fat pads were thymectomized at 8 weeks of age. A few weeks after thymectomy, solid pieces of living, immunizing, or control (normal virginal mammary gland or syngeneic antigenically distinct sarcoma) tissues were implanted intradermally. Immunizing tissues were removed surgically 14 days after implantation. Seven days after removal of the immunizing tissue, all animals were exposed to 400 R of whole-body X radiation. Within 24 hours after irradiation, challenge implants of solid pieces of living tissues were made into the 2 gland-free inguinal mammary



fat pads. This procedure was designed to interfere with the development of a primary immune response to the challenge tissues while leaving relatively intact the secondary response to antigens recognized before irradiation.

*Evaluation of growths.*—The skin, with attached mammary fat pads containing growths of experimental tissues, was pinned flat on corkboards and fixed in Bouin's solution, after which the growth of the then clearly visible mammary tissues was recorded. Selected specimens were removed from the skin, defatted in acetone, stained in Ehrlich's acid-hematoxylin, cleared in xylene, and mounted in histologic medium on glass slides for photography. Histologic sections of selected specimens, cut at 5  $\mu$ , were stained with hematoxylin and eosin.

*Statistical analysis of data.*—The 2-by-2 chi-square test with correction for continuity was used.

*Genotypically mosaic mammary glands.*—Mammary glands, composed of cells of 2 different genotypes originating from 2 different inbred strains of mice, were obtained in 2 ways: 1) Studies were done, in collaboration with Dr. Beatrice Mintz at this Institute, with tissues derived from her genotypically mosaic (allophenic) mice, and 2) mosaic mammary glands were produced by the association of adult mammary epithelial cells of 2 different strains. This association was accomplished by 2 methods: 1) Suspensions of mixtures of collagenase-dissociated cells were pelleted and inoculated into recipient gland-free fat pads, and 2) based on the results of Williams and Hoshino (4) and Chew and Hoshino (5), indicating that mammary gland transplants dissociate to single cells after transplantation and then regenerate mammary structures, mammary tissues of different genotypes were merely cut into small pieces, mixed, and then implanted into gland-free fat pads where they gave rise to genotypically mosaic growth. The genotypically mosaic mammary tissues were maintained by serial transplantation in F<sub>1</sub> hybrid hosts histocompatible with both "parental" genotypes. The cellular composition of these tissues and the growth characteristics of the component populations were determined by transplantation to the 2 inbred strains of origin. The histoincompatible population was rejected, and the histocompatible population gave rise to characteristic outgrowth. The symbol [//] will be used to designate the association of cells of different genotypes. Mosaic tissues com-

posed, *e.g.*, of B6 and C3Hf cells, are thus designated [B6//C3Hf].

*Terminology.*—Pren (6) has defined "neoplasia" as "that form of hyperplasia which is caused, at least in part, by an intrinsic heritable abnormality in the involved cells." Foulds (7) regarded the MTV-induced hyperplastic lesions of mice as forms of early neoplasia. I will use the term "neoplastic" in reference to populations of cells composed, at least in part, of cells having heritable alterations which cause the growth to be hyperplastic, otherwise morphologically abnormal, or predisposed to the development of malignancy.

Heritable alterations may be of various types or degrees and appear to be cumulative. The terms "less neoplastic" and "more neoplastic," therefore, refer to premalignant tissues tending to resemble normal growth and malignant carcinomas, respectively. A more neoplastic premalignant tissue might be one, *e.g.*, which, though grossly resembling malignant tissue, fails to exhibit malignant invasion.

## PREMALIGNANCY AND PROGRESSION

Outgrowth lines of premalignant tissues from lesions induced by MTV, MCA, hormone stimulation, or these agents combined, or from lesions arising in the apparent absence of oncogenic agents fell into 3 broad groups: those simulating normal mammary gland during pregnancy or lactation—"adenoid or alveolar growths," those made up of abnormal ductal structures—"ductal growths," and those exhibiting epidermoid or mesenchymal metaplasia—"squamous and mixed lesions." Adenoid premalignancies were induced by MTV, prolactin stimulation, these agents combined, or either of these factors plus MCA. Ductal lesions were derived from animals treated with MCA and MCA plus MTV. Characteristically, squamous and mixed lesions arise spontaneously late in life without known oncogenic agents, but they may be induced early in life and at a high frequency by MCA, apparently acting without other oncogenic factors. Such lesions also represent a proportion of those arising after MCA treatment of mice with MTV or hormonal stimulation and they arise spontaneously late in life in strains such as C3Hf, having a low virulence form of MTV (MTV-L).

Outgrowth lines of premalignant tissues derived from primary lesions within each of these 3 groups



represented growth types ranging between normal and malignant. Within each outgrowth line of virtually every tissue, neoplastic progression to malignancy occurred sequentially. Less neoplastic forms gave rise to grossly more neoplastic focal variants which, when transplanted to gland-free fat pads, grew by the extension of organized structures through the fat pad, and filled and considerably expanded the fat pad, producing growth grossly more neoplastic than the original or precursor form but not capable of malignant invasion or unlimited growth (fig. 1). These growths in turn gave rise to additional premalignant or malignant variants. Malignant variants did not grow by the extension of organized glandular structures, and they were not limited by the fascial connective tissues surrounding the fat pad or by the connective tissues investing vascular structures. Thus they invaded locally, metastasized, and rapidly killed the host.

### **MOSAICISM: ASSOCIATION OF SEPARATE POPULATIONS OF CELLS OF DIFFERENT TYPES IN PARENCHYMA OF GLANDS OF ECTODERMAL ORIGIN**

During the study of the antigenicity and response to immunity of premalignant tissues, I was surprised to find that, instead of being completely destroyed by the immune response, the premalignant tissues were changed into morphologically normal structures (fig. 3). When these normal-appearing tissues were transplanted serially in nonimmunized hosts, they continued to give rise to normal growths.

To determine the nature and origin of the normal tissues derived from premalignant tissues, genotypically mosaic mammary glands were studied.

#### **Mosaicism in Normal Tissues**

Studies of the normal tissues derived from genotypically mosaic B6↔C3Hf mice revealed that the normal mammary gland is composed of separate populations of cells able to form ductal and alveolar structures. When normal mammary tissues of mosaic origin were implanted in B6 recipients, ducts with few alveoli invariably grew. In contrast, transplants of early generations of these tissues produced growths of short ducts with many alveoli in C3Hf recipients, whereas those of

later generations frequently produced only small alveolar growths in C3Hf hosts. Daniel *et al.* (8) found that the capacities for alveolar and ductal proliferation aged separately when normal mammary tissues were transplanted serially in environments that primarily stimulated alveolar or ductal proliferation. Specifically, normal mammary tissues transplanted serially in mice continuously stimulated by abnormally high levels of prolactin lost the capacity for alveolar proliferation, whereas tissues transplanted serially in virgin hosts exhibited diminished capacity for ductal growth while retaining the capacity for normal alveolar proliferation.

Normal mammary glandular structures, both ducts and alveoli, are composed of 2 histologically different types of cells: the basally located myoepithelial cells and the luminal epithelial cells. These studies of the genotypically mosaic normal tissues and the studies of Daniel *et al.* indicate that normal mammary glands are composed of at least 3 separate populations of parenchymal cells representing 3 different cell types: 1) myoepithelial cells enveloping both ducts and alveoli (9, 10); 2) alveolar epithelial cells which, in association with myoepithelial cells, form alveolar structures; and 3) ductal epithelial cells which, in association with myoepithelial cells, form ductal structures. These 3 cell types appear to have separate origin in adult tissues.

#### **Mosaicism in Premalignant Tissues**

Adenoid premalignant tissues consisted of neoplastic alveolar epithelial cells associated with all 3 cell types of normal mammary gland cells. Several outgrowth lines of ducts with hyperplastic alveoli were derived from lesions induced by MTV in [B6//C3Hf] tissues and from lesions induced by MTV or MCA plus MTV in [C//C3Hf] tissues (fig. 4). Transplants of these premalignant tissues to B6 and C recipients, respectively, produced normal mammary-gland growths of ducts with alveoli, whereas transplants of these tissues to C3Hf recipients produced growth of short ducts with hyperplastic alveoli or hyperplastic alveolar structures lacking apparent basic ductal structure. Thus these growths of ducts with hyperplastic alveoli appear to contain all the components of the normal mammary gland of the B6 or C genotypes and variable amounts of the normal components of the

C3Hf genotype associated with neoplastic C3Hf alveolar epithelial cells.

Two ductal premalignant lines consisting of abnormal ductal structures lacking alveolar differentiation and frequently exhibiting papillary luminal projections (fig. 5) were studied. When the neoplastic component of these tissues was rejected by immunity, growth of normal ducts lacking alveoli resulted. Thus these ductal lesions may be composed of neoplastic ductal epithelial cells associated with normal myoepithelial and ductal epithelial cells and they may lack a significant component of alveolar epithelial cells.

MCA treatment of C3Hf, C, (C×D2)<sup>-</sup>, (C×D2)<sup>+</sup>, or (C×C3H) mice produced early in life a high incidence of premalignant lesions exhibiting squamous metaplasia. Transplants of these tissues gave rise to abnormal growth of fine, dense, frequently branching structures resembling no component of normal mammary gland (fig. 6). Squamous metaplasia frequently developed in these tissues. When these tissues were rejected in syngeneic hosts by an immune response directed against their neoantigens, no normal growth persisted. Similarly, such tissues, induced by MCA in genotypically mosaic tissues composed of [C//C3Hf] cells, produced typical squamous growth in either C or C3Hf recipients but no growths in the opposite strain. These tissues may consist of populations of neoplastic myoepithelial cells lacking normal cell components.

Whereas tissues exhibiting squamous metaplasia generally were nonmosaic, some types of tissues exhibiting squamous or mesenchymal metaplasia (e.g., fibroadenomas, mixed tumors) were composed of 2 or more types of parenchymal cells. Using electron microscopic and histochemical techniques, several investigators found evidence of 2 or more cell types, identified as myoepithelial and epithelial, in fibroadenomas and mixed tumors (10-14). These cell types have generally been assumed to result from divergent differentiation from a common neoplastic stem cell population (7). The myoepithelial cells comprising the major component of the parenchyma appear to produce the abnormal stromal fibrous, cartilaginous, or osseous tissues. From serially transplanted normal mammary-gland tissues composed of [C//D2] cells, I obtained, apparently without mammary oncogenic agents, a fibroadenoma (fig. 7). Transplants of this tissue produced rapidly growing fibroadenomas in

(C×D2)<sup>-</sup> hosts. In C recipients, these tissues grew at only about 0.1% of their growth rate in the F<sub>1</sub> hybrid and produced tiny tumor nodules which sometimes exhibited squamous metaplasia. Transplants of the fibroadenoma tissue to D2 recipients gave rise to entirely normal mammary-gland outgrowth. Thus fibroadenomas and similar lesions showing mesenchymal metaplasia may be composed of neoplastic myoepithelial cells associated with all 3 cellular components of normal mammary gland in approximately normal proportions. The normal cellular components interact synergistically with neoplastic myoepithelial cells to produce the rapid growth.

### Requirement for Association of Normal Cells With Neoplastic Cells During Progression to Malignancy

A more neoplastic, premalignant variant tumor tissue was derived from the [B6//C3Hf] lines. Transplants of this tissue produced normal growth in B6 recipients, whereas those in C3Hf recipients produced slowly growing hyperplastic structures which did not reproduce the growth rate or character of the original mosaic tumor (3). The reassociation of these small C3Hf growths with normal mammary gland tissues from young-adult B6 females resulted in the reproduction of the original mosaic tumor growth character in F<sub>1</sub> hybrid hosts. Transplants of these reassociated tissues from F<sub>1</sub> hybrid hosts produced typical normal growth in B6 but no growth in C3Hf recipients.

In histologic sections of mature specimens of the original mosaic tumor growths in F<sub>1</sub> hybrid hosts (though not in rapidly growing tissues early after transplantation), 2 different types of cells were clearly distinguished: basally located myoepithelial cells appearing cytologically normal and luminal neoplastic-appearing epithelial cells (3). Similar normal and neoplastic cell types were sometimes distinguishable in the original outgrowths in C3Hf hosts. Any C3Hf normal cell components in these structures would be aged 7 years and might show diminished growth potential as do serially transplanted aged normal mammary glands (8). Reassociation of these C3Hf tissues with young B6 glands placed the aged C3Hf normal cells in competition with young B6 normal cells and probably resulted in the replacement of the normal C3Hf components by B6 cells. The neo-



plastic C3Hf cells then failed to survive when returned to C3Hf hosts, possibly because they required association with normal parenchymal cells for survival. Transplants of malignant variants from the original mosaic tumor growth or from reassociated growths directly gave rise to carcinomas in C3Hf as well as in F<sub>1</sub> hybrid recipients, indicating that the malignant cells had lost the requirement for association with normal parenchymal cells.

In summary, these studies indicate that glands of ectodermal origin are composed of separate populations of parenchymal cells representing different cell types with separate origin in adult tissues. Premalignant lesions of these glands result from the neoplastic transformation of any one of the separate cell types. The neoplastic premalignant cells of each type form a characteristic class of lesions in which the neoplastic cells may require association with normal parenchymal cells. This requirement for cell-type associations may be a characteristic of the normal tissues which persists during neoplastic progression until malignancy develops. Malignant growth characteristics may result at least partly from the loss of dependence on association with particular normal cells. The various cell types making up the premalignant lesions and their derivation may not be readily determined by means of regular histologic techniques. Transplantation studies using genotypically mosaic tissues facilitate analysis of cellular composition and interactions.

## ANTIGENICITY OF MCA-INDUCED PREMALIGNANT AND DERIVATIVE MALIGNANT MAMMARY TISSUES

### Antigenicity of Premalignant Lines

*(C × C3H)<sup>Sea</sup> tissues.*—A premalignant outgrowth line (line 7) was derived from a lesion present in a *(C × C3H)<sup>Sea</sup>* mouse 34 weeks after the last of 17 doses of MCA given at 2-day intervals. The tissue exhibited adenoid or alveolar differentiation with units lined by single or double layers of cells frequently producing milk-like secretion (fig. 2). Three experiments were done to test the capacity of these tissues to induce and respond to transplantation resistance. The results are shown in table 1. They demonstrate that intradermal implantation of premalignant tissues of line 7

TABLE 1.—Effect of prior exposure to various tissues on resistance to transplants of *(C × C3H)<sup>Sea</sup>* line 7 premalignant tissue

Experiment No.	Immunizing tissue*	Number immune/total No.	P
1	Line 7	21/28	<0.02 Not significant
	Line 2	8/30	
2	Control	11/29	—
	Line 7	12/19	
3	Control	2/13	<0.01 — Not significant
	MTV-induced carcinomas	4/19	
	Control	5/26	

\* Intradermal implants of living tissues excised before challenge.

induced specific resistance to challenge with tissues derived from the same line, whereas immunization with premalignant tissues of a different syngeneic line (line 2) or carcinomas induced by MTV-L or MTV-S induced no resistance.

*(C × D2)<sup>-</sup> tissues.*—Three outgrowth lines exhibiting squamous metaplasia—lines 11, 13, and 16—were induced by MCA in *(C × D2)<sup>-</sup>* mice believed to lack active forms of mammary oncogenic viruses. Line 11 was transplanted from the MCA recipient donor 14 weeks after the last of 9 weekly doses of MCA. The line 13 primary lesion was found 4 weeks after the last of 17 doses of MCA given at 5-day intervals. Line 16 was isolated from a hyperplastic lesion present 9 months after transplantation of morphologically normal tissue from an MCA recipient 20 weeks after the last of 9 weekly doses of MCA.

Five experiments tested the antigenicity and response to specific immunity of the premalignant tissues of these 3 lines. The results of these experiments are summarized in table 2. They show that the premalignant tissues of each line exhibited non-cross-reacting, tumor-specific antigenicity; each line induced significant radiation-resistant immunity to challenge implants of tissues of the same but not of a different line.

One outgrowth line derived from an MCA-treated *(C × D2)<sup>-</sup>* mouse (line 15) exhibited abnormal ductal structure lacking squamous metaplasia and alveolar differentiation (fig. 5). One small experiment tested the antigenicity of this tissue. While the results were not statistically significant—6 of 10 immunized versus 2 of 10 control animals showing evidence of immunity,

TABLE 2.—Antigenicity of (C × D2)<sup>-</sup> lines 11, 13, and 16

Experiment No.	Immunizing tissue* line No.	Challenge tissue line No.	Number immune/total No.	P
4	11	11	22/30	<0.001
	Control	11	0/15	—
5	13	13	11/32	<0.05
	Control	13	0/16	—
6	16	16	11/25	<0.001
	Control	16	0/26	—
7	16†	16‡	25/40	<0.001
		11	6/40	>0.1
	Control	16	0/29	—
		11	1/29	—
		11‡	25/26	<0.001
8	11	13	0/26	—
		11	0/30	—
	13	13	22/30	<0.001
	Control	11	1/30	—
		13	2/29	—

\* Intradermal implants of living tissues excised before challenge.

† Hyperimmunized; 2 intradermal inoculations of experimental or control tissues spaced 14 days apart.

‡ Each animal received a challenge implant of line 11 in 1 gland-free inguinal fat pad and line 16 or 13 in the other.

$P > 0.05$ —the observation of normal outgrowths and patterns of mixed normal and hyperplastic growth consistent with immunologic rejection, as observed in other lines, strongly indicated that this tissue was antigenic and contained components of normal cells.

(C × D2)<sup>+</sup> tissues.—Ten outgrowth lines, isolated from lesions present in (C × D2)<sup>+</sup> mice 0–9 months after the administration of either 6 or 9 weekly doses of MCA, were studied for antigenicity and response to specific immunity. A separate experiment was done in immunized and control (C × D2)<sup>+</sup> and immunized (C × D2)<sup>-</sup> mice to test each of the 10 lines.

These experiments demonstrated that premalignant mammary tissue induced by MCA acting with MTV-S in (C × D2)<sup>+</sup> mice frequently exhibited immunogenicity in (C × D2)<sup>+</sup> mice. A total of 27 of 52 [52% of immunized (C × D2)<sup>+</sup> mice had specific immunity to the premalignant tissues of the various MCA-induced outgrowth lines versus only 3 of 53 (6%) nonimmunized controls ( $\chi^2 = 26$ ,  $P < 0.001$ ]. Of 52 immunized MTV-free (C × D2)<sup>-</sup> mice, 33 (63%) showed immunity. This figure did not differ significantly from that of 27 of 52. Thus lesions induced by MCA acting with MTV may have frequently expressed significant nonviral non-cross-reacting neoantigenicity. Lavrin *et al.* (15, 16) and Lavrin (17) showed that MTV-S-induced premalignant mammary tissues of C3H mice were not immunogenic in C3H

recipients. Similarly, Dezfulian *et al.* (18) found that MTV-S-induced tumor tissues from BALB/c mice fostered by C3H (CfC3H mice) were not immunogenic in CfC3H mice. Tissues induced by MTV-S are typically immunogenic only in MTV-S-free recipients. Several investigators found, however, that some MTV-S-induced tumors demonstrated, in addition to the MTV-S-associated antigenicity, tumor-specific, non-cross-reacting neoantigenicity (19–24).

From these published studies, the premalignant mammary tissues of (C × D2)<sup>+</sup> origin would be expected to exhibit little or no specific immunogenicity in (C × D2)<sup>+</sup> hosts unless the MCA treatment had influenced the development of neoantigenicity. The high incidence of immunogenicity observed therefore indicates that MCA influenced antigen induction or expression. Possibly, the expression of the type of neoantigenicity, which tends to arise spontaneously in MTV-S-induced tumors, was increased by MCA treatment. Alternatively, the studies of Mondal *et al.* (25) and of Basombrío (at this Conference) indicate that MCA may directly induce antigenicity.

Lesions induced by MCA plus MTV-S may resemble either MTV-induced lines (producing fine hyperplastic alveolar growth) or lesions induced by MCA in mice without MTV (exhibiting squamous metaplasia). The typical line, however, induced by MCA plus MTV-S, appeared to exhibit



growth characteristics reflecting the influence of both agents (large, dense, frequently fibrous growths with alveolar differentiation containing a component of normal cells and expressing strong neoantigenicity). Two adenoid lines and three squamous lines had high antigenicity, whereas one adenoid and three squamous lines had low antigenicity. One ductal line was not detectably antigenic, but a previous test of the (C × D2)<sup>-</sup> ductal line (line 15) indicated that such tissues may be antigenic. Thus there was no correlation between cell type of origin and the expression of neoantigenicity.

**Relationship of Antigenic Strength and Specificity of Premalignant Tissues to Those of Derivative Malignant Tissues**

Two experiments tested the antigenic strength and specificity of malignant variants derived from premalignant lines known to express neoantigenicity. One experiment tested (C × C3H)<sup>Sea</sup> line 7, and the other, (C × D2)<sup>-</sup> line 11. In the first experiment, the immunizing and challenge tissues were derived from different sublines having common origin 3 years before the experiment. The results of these experiments are summarized in table 3. They indicated that premalignant and derivative malignant tissues had similar antigenic strength and specificity. This was true for both the alveolar epithelial type (line 7) and the myo-epithelial type (line 11) of neoplasia. Both premalignant and derivative malignant immunizing tissues induced a high incidence of strong cross-

reacting immunity to challenge with either premalignant or malignant tissues from the same line. The study of the bilateral correlation of immune response supported the concept that the related premalignant and malignant tissues have similar antigenicity. Never were malignant challenge implants rejected while premalignant tissues survived; all 71 animals rejecting the malignant challenge implants also rejected the premalignant tissue. In all 18 cases where premalignant growth occurred in immunized animals, malignant growth also was not inhibited. There were 21 mice, however, in which premalignant implants were rejected while some malignant growth continued. In 10 of these, however, the tumors were smaller than the smallest of the tumors in the control animals. Therefore, there was some effective immunity early after malignant challenge in these animals, but malignant growth could persist while the premalignant tissue was destroyed.

While these experiments were in progress, other investigations in Dr. Prehn's laboratory by Lappé (26, 27) and Lappé and Prehn (28) demonstrated the expression of neoantigenicity by chemical oncogen-induced premalignant skin lesions. Lappé (29) also found evidence that malignant variants from the antigenic premalignant lesions shared antigenic specificity with the precursor premalignant tissues.

These observations indicate that the specific neoantigenicity commonly observed in malignancies induced by chemical oncogens may develop early in the process of oncogenesis and persist essentially unchanged through years of serial

TABLE 3.—Antigenicity of premalignant (Prem) and derivative malignant carcinoma (M) tissues

Experiment No.	Line No. and genotype	Immunizing tissue*	Challenge tissue	Number immune/total No.	P
9	7(C × C3H) <sup>Sea</sup>	Prem	Prem†	12/20	<0.02
			M	10/20	<0.005
		M	Prem	12/18	<0.01
			M	12/18	<0.001
		Control	Prem	2/15	—
		(sarcoma)	M	0/15	—
10	11(C × D2) <sup>-</sup>	Prem	Prem	28/29	<0.001
			M	22/29	<0.001
		M	Prem	31/32	<0.001
			M	27/32	<0.001
		Control	Prem	0/29	—
			M	0/29	—

\* Intradermal implants of living tissues excised before challenge.  
† Each animal received an implant of premalignant tissue in 1 gland-free inguinal fat pad and of malignant tissue in the other.

transplantation and during progression to malignancy. The premalignant tissues expressed the strong non-cross-reacting, tumor-specific neoantigenicity characteristic of chemical oncogen-induced malignant neoplasms (30).

### IMMUNITY—HOST RESPONSE TO ANTIGENIC PREMALIGNANT AND DERIVATIVE MALIGNANT MAMMARY LESIONS

If the early lesions arising in the breast express strong specific antigenicity, persist, and give rise to antigenic malignant variants which kill the host, it must be that the host remains unaware of the presence of the lesions or is failing to mount an *effective* immune response at least while the lesions are small, slowly growing, and susceptible to immune destruction.

#### Host Response to Simulated *In Situ* Lesions

The study of immune response to primary lesions *in situ* is complicated by the fact that each lesion probably expresses unique antigenicity, and no method is yet known for immunization against them. Transplanted tissues having known antigenicity can, however, be made to simulate primary lesions if they are implanted in mammary fat pads containing normal host epithelium. Their outgrowth is inhibited by the normal tissues, but they may expand slightly to form lesions simulating primary lesions *in situ*.

Two experiments were done to test the host response to simulated *in situ* antigenic lesions. The first experiment tested the response to bilateral implants of the highly antigenic (C × D2)–line 11 tissues. The second experiment tested the response to simultaneous implants of line 11 tissues and to the more neoplastic, less antigenic tissues of (C × D2)–line 16.

First, “challenge” implants were made into the normal host glands. Then after 8–12 weeks had elapsed to allow for recovery from the surgery and to allow the lesion to develop to simulate a primary lesion, the animals were immunized by intradermal implantation of experimental premalignant tissues or control normal tissues. In the first experiment, the animals received one immunizing inoculation. In the second experiment, the animals received 2 inoculations spaced at an interval of 14 days (hyperimmunization).

The results of these experiments are summarized

in table 4. They indicate that premalignant lesions arising in the mammary gland, though highly antigenic, fail to immunize the host but remain, however, antigenic and susceptible to immune destruction. Immunization may be delayed until the lesions have progressed to stages more rapidly growing, greater in mass, and perhaps capable of overriding or circumventing the immune response. Most animals hyperimmunized against line 16 could reject subsequent challenge implants of line 16 tissues (expt. 7). When the line 16 tissue was allowed to grow *in situ*, however, immune destruction was not accomplished by hyperimmunization. During growth *in situ*, the mass increased from the implanted mass of about 0.2 mg to 10–75 mg. Since the immune response to line 16 was relatively weak, *i.e.*, capable of destroying 0.2 mg in about 80% of mice hyperimmunized before challenge, the increased mass may have overwhelmed the immune capacity. Simulated *in situ* line 11 premalignant lesions increased from an implanted mass of about 0.5 mg to 5–25 mg after 3 months, but remained completely susceptible to immune destruction.

#### Response of Immunized Recipients to Challenge Implants of Antigenic Premalignant or Derivative Malignant Tissue

*Mechanisms of rejection of premalignant and malignant tissues.*—In experiments designed to study the antigenicity of malignant tissues in general, complete destruction, decreased size of tumors after a given period of time, or a decreased growth rate of the tumors may be observed. I expected to see any of these effects, particularly destruction or decreased growth rate in immunized animals when the premalignant tissues were implanted. However, instead of being completely destroyed or exhibiting a decreased growth rate, the implanted antigenic premalignant tissues grew at a normal rate in immunized animals if they were not immediately rejected. Then, beginning at the transplantation site and progressing slowly over a period of weeks or months toward the growing periphery, a cellular infiltration was associated with the destruction of the antigenic premalignant cells, whereas the normal cells were left undamaged to form normal structures (fig. 3). This partial rejection progressed slowly toward the periphery of the outgrowth at different rates in different animals. Sometimes the infiltrating cells overtook the growing hyperplastic

TABLE 4.—Host response to simulated *in situ* antigenic premalignant lesions

Experiment No.	Challenge tissue line No.	Immunizing tissue*	Number immune/total No.	P
11	11	11	19/19	<0.001
	11	Normal	0/20	—
12	11†	11‡	23/23	<0.001
	16		0/23	—
	11	16‡	3/23	Not significant
	16		2/23	Not significant
	11	Normal‡	2/25	—
	16		0/25	—

\* Intradermal implants of living tissues excised 14 days after implantation.  
† Each animal received an implant of line-11 tissue in 1 inguinal mammary gland and line-16 tissue in the other.  
‡ Hyperimmunized.

structures after several weeks and accomplished the complete rejection of all neoplastic cells. In other cases, the partial rejection reaction progressed for a time and then slowed or stopped, and premalignant tissue persisted and grew indefinitely.

The decline in effective immunity could have resulted from the rate of premalignant growth exceeding the rate of rejection, with the consequent increased mass of antigenic tissue overwhelming the immune response. Alternatively, a decreased antigenic stimulus, gradual exhaustion of immune capacity, or the induction of blocking antibody could account for these observations.

*Hiding of neoplastic cells within normal structures.*—Serially transplanted tissues derived from transplants of morphologically normal structures left after complete rejection of line 7 tissues remained normal through 6 transplant generations. Serially transplanted tissues derived from morphologically normal areas of a partially rejected outgrowth (fig. 3) produced normal outgrowth in the first and second transplant generations. In the third and fourth generations of 1 of 3 sublines of 1 of these lines, however, hyperplastic lesions arose. These lesions, and the serially transplanted lines derived from them, greatly resembled the original line 7 tissues. Although the antigenic strength and specificity of these tissues have not been tested, premalignant tissues resembling line 7 rarely, if ever, occur spontaneously and these hyperplastic tissues probably represent populations of antigenic neoplastic premalignant cells of line 7 which persisted within the normal structures possibly protected there from immune destruction.

*Relative susceptibility of premalignant and malignant tissues to immune destruction.*—In the experiments

testing the antigenicity of premalignant and derivative malignant tissues (expts. 9, 10), the malignant tissues seemed relatively resistant to immunity as compared with the premalignant tissue of origin, even though the antigenic strength and specificity of the 2 tissues appeared similar. This could have been caused by mass effects resulting from the rapid growth of the malignant tissues, or it could have resulted from the relative resistance of the malignant cells to the cytotoxic effects of immune cells.

Transplants of the genotypically mosaic [C//D-2] fibroadenoma, discussed above, occasionally gave rise to genotypically mosaic fibroadenoma growth in C or D2 recipients. Transplants of such tissues to nonimmunized C and D2 recipients produced the tiny tumor growths in the former and the typical normal growths in the latter. More commonly, the premalignant C component was rejected in the D2 recipients of mosaic growths from F<sub>1</sub> hybrid donors, and normal growth persisted. However, 3–6 months later, malignant growths sometimes arose. When this occurred, the malignant growths frequently developed simultaneously in both recipient fat pads. Transplants of these malignant growths produced no growth in D2 recipients but malignant growth in C recipients. These observations indicate that in the case where normal or neoplastic cells within the fibroadenoma transplant were usually, but not always rejected, when premalignant cells were rejected, malignant cells sometimes survived. The malignant cells, though their antigenic specificity was the same as that of the premalignant cells, were relatively resistant to immune destruction. The antigenic malignant cells were apparently controlled for



variable periods by systemic immunity but eventually escaped and produced lethal growth.

## DISCUSSION

Antigenic premalignant lesions arising in the mammary gland fail to alert the host to their presence and thus do not induce an effective immune response. Billingham and Medawar (37) and Billingham and Silvers (32-34) showed that allogeneic melanocytes transplanted into the guinea pig epidermis may survive and grow indefinitely, failing to immunize the host. Woodruff and Woodruff (35) and Merwin and Hill (36) found that allogeneic Harderian and thyroid gland implanted in the loose subcutaneous connective tissues persisted for long periods, apparently failing to immunize. Moretti and Blair (37) and Blair and Moretti (38, 39) found that normal B6 male mammary tissues expressing the male antigen survived and grew in female B6 hosts when implanted in gland-free fat pads, apparently not inducing effective immunity.

The antigenic premalignant mammary tissues, allogeneic melanocytes, allogeneic glandular tissues, and male mammary tissues have in common the separation of the antigenic cells from the host by a basement membrane. Perhaps as long as the basement membrane remains intact, antigen may not contact appropriate cells or reach appropriate sites to initiate the development of effective immunity. The normal or neoplastic cells in transplants of the genotypically mosaic fibroadenoma may sometimes survive in allogeneic hosts partly because they fail to immunize effectively. The fibrous stroma of the fibroadenoma, representing abnormal amounts of basement-membrane material produced by the neoplastic myoepithelial cells, may serve to block the afferent limb of the immune response by hiding the cells from host recognition processes. The survival of malignant cells when premalignant cells were destroyed indicates a defect in the efferent limb as well. The malignant cells may be relatively resistant to the cytotoxic effects of immune cells or they may be able to survive in sites immune cells cannot reach or in which immune cells cannot survive or function.

Antigenic premalignant lesions may fail to immunize until progression to malignancy (with invasion of or loss of functional basement mem-

brane) has occurred, and then the lesions may be relatively less susceptible to immune destruction. These observations question the efficacy of natural immunosurveillance as a defense against these types of neoplasia.

Premalignant tissues, both when simulating *in situ* lesions and when growing in gland-free fat pads, were rejected by a cellular infiltration that was initiated at the site of origin of the lesion and progressed slowly toward the periphery. This reaction progressing for weeks or months could eventually destroy all neoplastic premalignant cells. Such a reaction would not be effective against a more rapidly growing or larger premalignant or malignant mass. In addition, differences in relationships of the parenchymal cells to each other and to the stroma, whence the immune cells enter, may be related to the relative resistance of the more neoplastic tissues to immune destruction. The entire mass may have to be invaded simultaneously, or immune destruction may occur primarily on the periphery of the mass. As a consequence, a much stronger immune response is necessary to effect complete destruction.

When antigenic premalignant mammary tissues were implanted in immunized recipients, the tissues were sometimes only partially destroyed before the immune response diminished and premalignant growth continued. Billingham and Medawar (37) observed that allogeneic melanocytes growing in the epidermis sometimes immunized the host, were then partially destroyed by the immune response, but recovered and continued their growth. If a relatively weak immune response was generated by brief application of specific antigen, these investigators noted a similar temporary partial rejection of the allogeneic cells. Blair and Moretti (39) noted lymphoid infiltration associated with the growing antigenic mammary tissues, but rejection did not occur. Clark and Mihm (40) and Clark *et al.* (41) observed that premalignant lesions of melanocytes in humans apparently underwent prolonged partial regression phenomena mediated by classical cellular immunity. The antigenic premalignant mammary tissues, allogeneic melanocytes, and allogeneic Harderian and thyroid glands retained their antigenic specificity and were rapidly and completely destroyed when a specific immune response was induced against them.

These observations indicate that antigenic

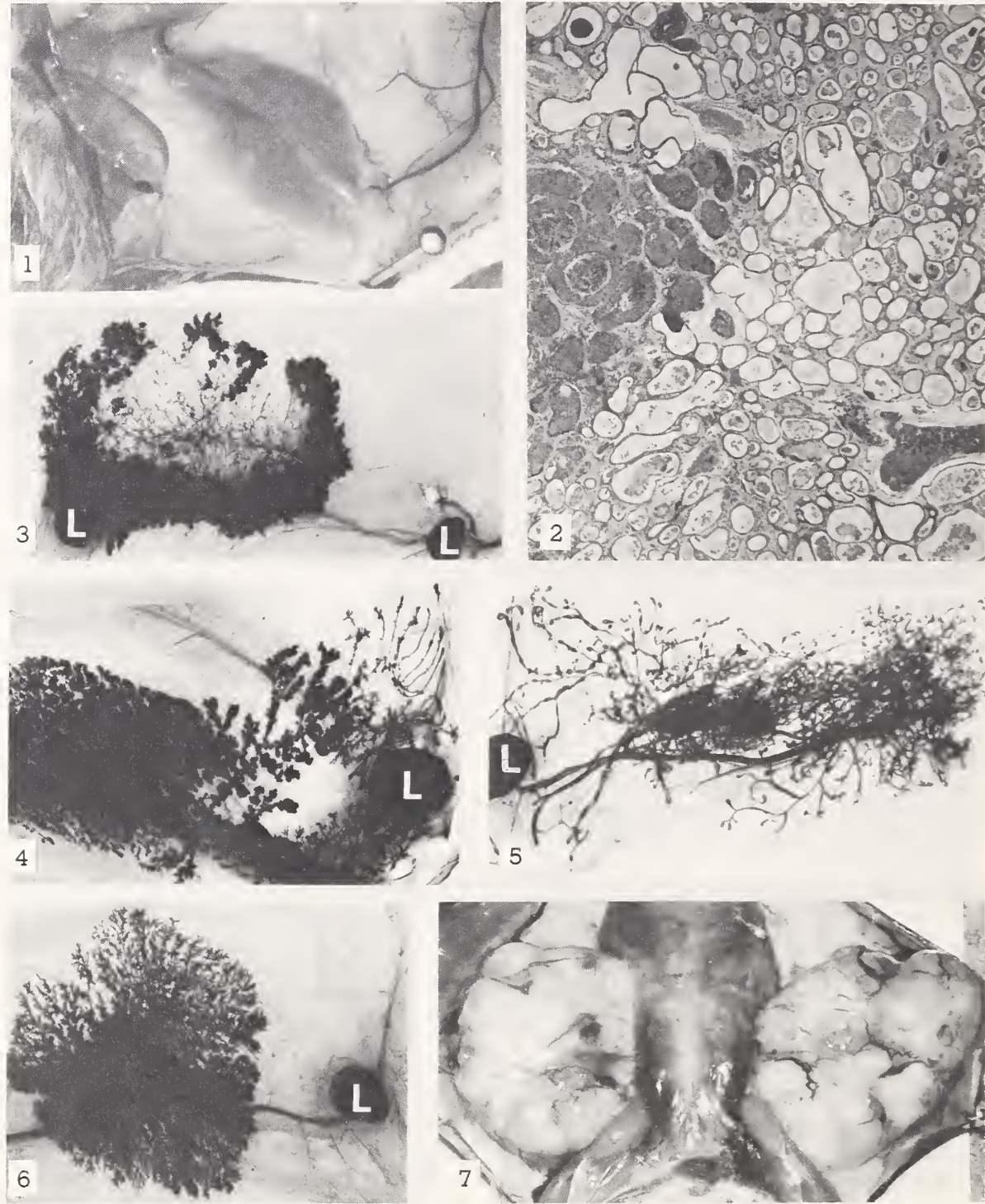




FIGURE 1.—Typical adenoid premalignant tissue representing a stage intermediate between typical precursor forms and malignant carcinoma. Growth *in vivo* 12 weeks after transplantation extends to fill, conforms to, remains confined to, and considerably expands the mammary fat pad.

FIGURE 2.—Photomicrograph of hematoxylin and eosin-stained histologic section of typical adenoid premalignant tissue with a malignant variant 56 weeks after transplantation. Premalignant structures, lined by thin layers of epithelium, produce milk-like secretion and give rise to malignant variants composed of multilayers of cells capable of invading adjacent premalignant tissues, stroma, and vascular structures.

FIGURE 3.—Hematoxylin-stained wholemount preparation of adenoid premalignant tissue partially rejected by a specific immune response 14 weeks after implantation. Normal structures persist around transplantation site centrally while neoplastic growth continues on periphery. L = lymph nodes in each wholemount. Dark structures crossing lymph nodes are blood vessels.

FIGURE 4.—Portion of a wholemount of typical precursor adenoid premalignant ducts with hyperplastic alveoli 88 weeks after implantation. These tissues consist of neoplastic alveolar epithelial cells associated with all 3 normal mammary gland components.

FIGURE 5.—Wholemount of ductal premalignant tissue 6 weeks after implantation. This tissue may be composed of neoplastic ductal epithelial cells associated with normal myoepithelial and ductal epithelial cells.

FIGURE 6.—Wholemount of typical, nonmosaic, squamous-type, premalignant tissue 8 weeks after implantation. Fine, frequently branching structures corresponding to no component of normal mammary gland may consist of neoplastic myoepithelial cells lacking normal cell components.

FIGURE 7.—Growth *in vivo* of genotypically mosaic [C//D2] fibroadenoma composed of neoplastic C myoepithelial cells associated with all 3 types of normal D2 mammary gland cells.



tissues—especially those protected by basement membrane—frequently failed to immunize and, when they did, the response sometimes diminished before effecting complete destruction, even though the tissues remained highly antigenic and susceptible to destruction and the host was fully capable of mounting an effective response to appropriate antigenic stimulation. Diminution of antigenic stimulus or the formation of blocking factors could account for these observations. I favor the former hypothesis and believe these observations indicate that the immune response to antigenic lesions *in situ* is frequently much less than the maximum possible. This may be true for malignant as well as for premalignant lesions.

Baillif (42) and Baillif and Jones (43) observed that, in immunized mice, a population of Ehrlich carcinoma cells was sometimes only partially destroyed by immunity. Some malignant cells persisted, apparently in sites protected from the immune response; months later they produced lethal growth apparently after exhaustion of the immune response. The late appearance of the allogeneic malignant tumors I observed in recipients of the mosaic fibroadenoma may result from a similar phenomenon.

The phenomena of failure to immunize maximally, of immunoselection for less antigenic variants in neoplastic cell populations (44, 45), of immune exhaustion, the relative resistance of more neoplastic cells to immune destruction, as well as simple mass effects indicate that immunotherapy of neoplasia is most likely to be effective in destroying all the neoplastic cells if a maximal immune response is generated as early as possible by a specific immunization procedure.

The observation of cross-reactions between neoplasms of similar type which Dr. Ingegerd Hellström will discuss (in this Conference), the demonstration of similar antigenicity in premalignant and derivative malignant tissues reported here, and the observations of apparent effective cross-reactivity between premalignant and malignant lesions of human breast that Dr. Black will describe (in this Conference) suggest that methods may be found for the effective immunoprophylaxis of malignancy.

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# Cellular and Biologic Manifestations of Immunogenicity in Precancerous Mastopathy<sup>1</sup>

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**SUMMARY**—Previous studies from this laboratory had identified the prognostically favorable significance of various lymphoreticuloendothelial responses in breast cancer patients: lymphoid cell infiltration and perivenous lymphoid cell infiltration in the primary tumor, sinus histiocytosis in the axillary nodes, and specific types of cellular response to autologous cancer tissue on skin window testing. In the present study, these responses appeared to reflect cellular hypersensitivity. They were particularly frequent in *in situ* breast carcinomas. Moreover, it appeared that *in situ* carcinoma provoked a cross-reacting immune response against the progressive growth of subsequently developing breast cancer.—*Natl Cancer Inst Monogr* 35: 73–82, 1972.

IN 1950, Black, Bolker, and Kleiner reviewed the subject of morphology and metabolism in malignant neoplasia (1). In their report they noted that, “. . . at least in its initial stages, malignant neoplasia represents a delicate balance between the local growth and the milieu of the host.” In addition, they concluded that, “. . . further work on cancer as a systemic disease in general and the tumor-host relationship specifically appears justified at this time.” These conclusions prompted an investigation of structural responses in the lymphoreticuloendothelial (L-RE) system of cancer patients, with particular reference to axillary lymph nodes removed during mastectomy for breast cancer. The biologic significance of lymph node structure in breast cancer patients was initially reported in 1953 (2). Most pertinent was the finding that a distinctive pattern of lymph node response, sinus histiocytosis (SH), appeared to be prognostically significant. These observations were confirmed in a series of studies which further demonstrated that

the biologic behavior of breast cancer reflected an interaction between the aggressive potential of the cancer cells and protective responses of the host L-RE system (3–7). The former could be correlated with the nuclear grade of the cancer cells, while the latter could be related to SH responses in the axillary lymph nodes (8, 9). These studies demonstrated that the L-RE system is not indifferent to the presence of breast cancer. Furthermore, the structural features of the prognostically favorable SH responses appeared more consistent with a delayed type of hypersensitivity than with a hyperimmune (immediate) type of hypersensitivity (9–11). This interpretation was strengthened by the finding that perivenous lymphoid cell infiltrations (PVI's) were in the primary tumors of 60% of SH-positive patients whereas they were in the primary tumors of only 20% of SH-negative patients. It should be recalled that PVI is a hallmark of cellular hypersensitivity in tissues (12).

Another type of lymphoid cellular response should also be mentioned: diffuse lymphoid cellular infiltration (LI). It is associated with a minor percentage of invasive breast cancers. LI is most commonly observed in so-called medullary carcinomas. This association was emphasized by Moore and Foote who commented on the relatively favorable prognosis of such lesions (13). More

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fundamentally, LI is preferentially associated with breast cancers having low nuclear grades; *e.g.*, approximately 80% of breast cancers with LI have highly anaplastic nuclei. In contrast to the PVI response, which is predominantly lymphocytic, the lymphoid cell population of LI is commonly plasmacytic or mixed. Also LI in the primary cancer is usually associated with marked follicular hyperplasia in the axillary lymph nodes. An association of LI in the primary tumor and marked follicular hyperplasia in the regional lymph nodes has also been found to be prognostically favorable in gastric carcinoma (14-16).

Stewart and Orizaga reported on hypersensitivity responses to intradermal injections of extracts of autologous cancers in patients with breast cancers (17). The skin reaction was temporally and microscopically suggestive of a delayed type of hypersensitivity and was found more frequently among patients whose cancers showed LI and low nuclear grades. However, such skin reactivity was not correlated with superior survival. The precise immunologic interpretation of LI and the skin test of Stewart and Orizaga was not clear. However, Stewart and Orizaga suggested a mixed Arthus and delayed-hypersensitivity response, an interpretation consistent with the presence of plasma cells.

More recently Black and Leis (18, 19) described a skin window procedure for visualizing cellular responses to autologous cancer tissue. Responses of a type consistent with cellular hypersensitivity were observed in approximately one-third of patients with invasive breast cancer. Such responses are particularly frequent among patients having SH reactivity in their axillary lymph nodes and/or PVI's in the primary tumor.

Considered *in toto*, invasive breast cancer apparently provokes immunologic responses in the L-RE system of the host. Such responses are reflected in cellular changes in the nodes and primary tumor and are measurable in terms of the responses of circulating lymphoid cells. In short, immunologic responses constitute biologically significant components of the tumor-host relationship.

## RESULTS

### *In Situ* Carcinoma

The demonstration of immunogenicity in invasive breast cancer raises a question regarding chron-

ology: Does antigenic change occur *pari passu* with the malignant transformation or does it represent an epiphenomenon? A natural experiment for examining this question in man is provided by *in situ* carcinoma. Since *in situ* carcinomas represent the earliest recognizable form of breast cancer and since they lack invasion, they provide a more direct test of the relationship between malignant transformation and L-RE responses than do invasive cancers.

### Characteristics of *In Situ* Carcinoma

Before L-RE responses to *in situ* carcinoma are considered, the structural and biologic characteristics of *in situ* carcinoma of the breast should be defined. The usual connotation given to the designation "*in situ* carcinoma" is a lesion which has the cellular characteristics of carcinoma except for invasion, *i.e.*, a preinvasive carcinoma. The latter potential is inferred from two types of observations: 1) Such lesions are seen with some regularity close to invasive carcinoma and 2) minimally resected (biopsy only) *in situ* carcinomas are followed by an increased frequency of subsequently appearing carcinomas of the tissue in question. However, the subsequent cancers may be *in situ* or invasive and may occur in the opposite breast almost as frequently as in the homolateral breast. Furthermore, they occur in only 25-35% of patients with biopsy-treated *in situ* carcinoma. While far from invariable, such an incidence rate is at least 5 times greater than the expected incidence among age-matched controls. In this regard, *in situ* carcinoma of the breast fits the definition of a precancerous lesion.

To define the structural characteristics of *in situ* carcinoma more precisely, Black and Chabon devised a numerical grading system for assessing the degree of atypia occurring in different divisions of the mammary duct system (20). The grade values were defined as follows: grade 1—normal-appearing duct epithelium; grade 2—normotypic hyperplasia; grade 3—epithelial atypia, minimal; grade 4—epithelial atypia, moderate; and grade 5—epithelial atypia, marked. The grade 5 changes were of the type conventionally designated as *in situ* carcinoma. Such grading was assigned individually to each of the major subdivisions of the branching mammary duct system: A—larger interlobular ducts; B—terminal interlobular ducts; C—initial segment of intralobular ducts; and

TABLE 1.—Percent incidence of duct grades 3–5 in “benign” breast biopsies

Group	Ducts					
	Apocrine	A	B	C	D	A–D
Unselected (207)	10	6	10	6	3	12
Non-precancerous (262)	7	2	8	3	2	9
Precancerous (92)	19	20	34	22	20	46

D—terminal portion of intralobular ducts. Also noted were areas of apocrine metaplasia which are also graded as above. It is thus possible to designate the degree and duct location of *in situ* atypical changes in a simple fashion.

To assess the biologic significance of various degrees of atypia, Black *et al.* examined the duct grades in 2 series of “benign” biopsies with prolonged follow-up (21). In one series, the biopsy was followed by the occurrence of cancer. In the other series, no cancer was observed within 10 or more years. It will be seen from table 1 that breast lesions preceding the occurrence of cancer were characterized by a greater incidence of atypia in all duct divisions than was found in unselected series and in the non-precancer group. Also, duct atypia  $\geq 3$  was seen near invasive breast cancer in more than 60% of cancerous breasts. More detailed analysis of the data in table 1 disclosed that atypia, grades 3 and 4, was associated with a fivefold greater risk of development of subsequent breast cancer than nonatypical benign lesions. However, the cancer might not occur for 10–20 years after the initial atypical lesion and might appear in the opposite breast in approximately one-third of the cases. These findings warrant the general designation of precancerous mastopathy (PCM) to include the range of duct grades 3–5. Therefore, in the immunologic investigations of mammary carcinogenesis, one should include the range of lesions from grade 3 atypia through invasive carcinoma.

## L-RE Responses to PCM

### *Intraepithelial Lymphocyte Accumulation*

Before the question of the more conventional types of L-RE responses to PCM is examined, note should be taken of a much overlooked facet of lymphocyte-epithelial cell interaction. Anatomists

have long been aware that lymphocytes migrate in and among the epithelial cells of diverse mucous membranes, a phenomenon designated emperipolesis (22). Black and Chabon noted that intra-epithelial lymphocyte accumulations (ILA) were regularly seen in normal mammary ducts, in areas of normotypic apocrine metaplasia, in hyperplastic foci (duct grade 2), and in fibroadenomas (20, 23). In contrast, ILA were characteristically diminished or absent in areas of *in situ* carcinoma (duct grade 5). Diminished ILA were also found in duct grades 3 and 4. While one may occasionally observe ILA even in grade 5 lesions, the usual observations are depicted in figures 1–4, wherein lymphocytes are seen in the hyperplastic duct epithelium ( $B_2 C_2 D_2$ ) but not in duct grade 4 lesions ( $B_4 C_4 D_4$ ).

Apparently PCM is associated with a change in the physiologic relationship between lymphocytes and epithelial cells. In terms of current interests, one might suggest that ILA are representative of immunologic surveillance and interpret the loss of ILA in foci of PCM as a loss of surveillance function which favors the development of neoplasia. However, the loss of ILA in foci of PCM is not associated with a similar change in adjacent normotypic or hyperplastic ducts, wherein normal or even increased ILA prominence is usual. It appears more reasonable at this time to interpret the observations as indicating a subtle change in the epithelial cells which either decreases their lymphocytotactic properties or a stromal cell change which impedes the entrance of lymphocytes into epithelial membranes. What is clear is the need for further investigation of the physiologic function of ILA and the basis of the alteration observed in the early phases of carcinogenesis.

The current preoccupation with cancer immunology should not lead to the unwarranted assumption that all changes in epithelial-stromal cell relationships involve immunologic phenomena. The basic mechanisms involved in epithelial-stromal cell interactions in development, repair, and hyperplasia are still unknown and may well be pertinent to the earliest phases of carcinogenesis. In this connection, some interesting observations were made on mast cells in regions of experimentally induced precancerous lesions. Localized increases in the number of mast cells were demonstrated in association with precancerous proliferations of epithelium in several sites (including breast) in rats and mice. This accumulation was reversed upon development



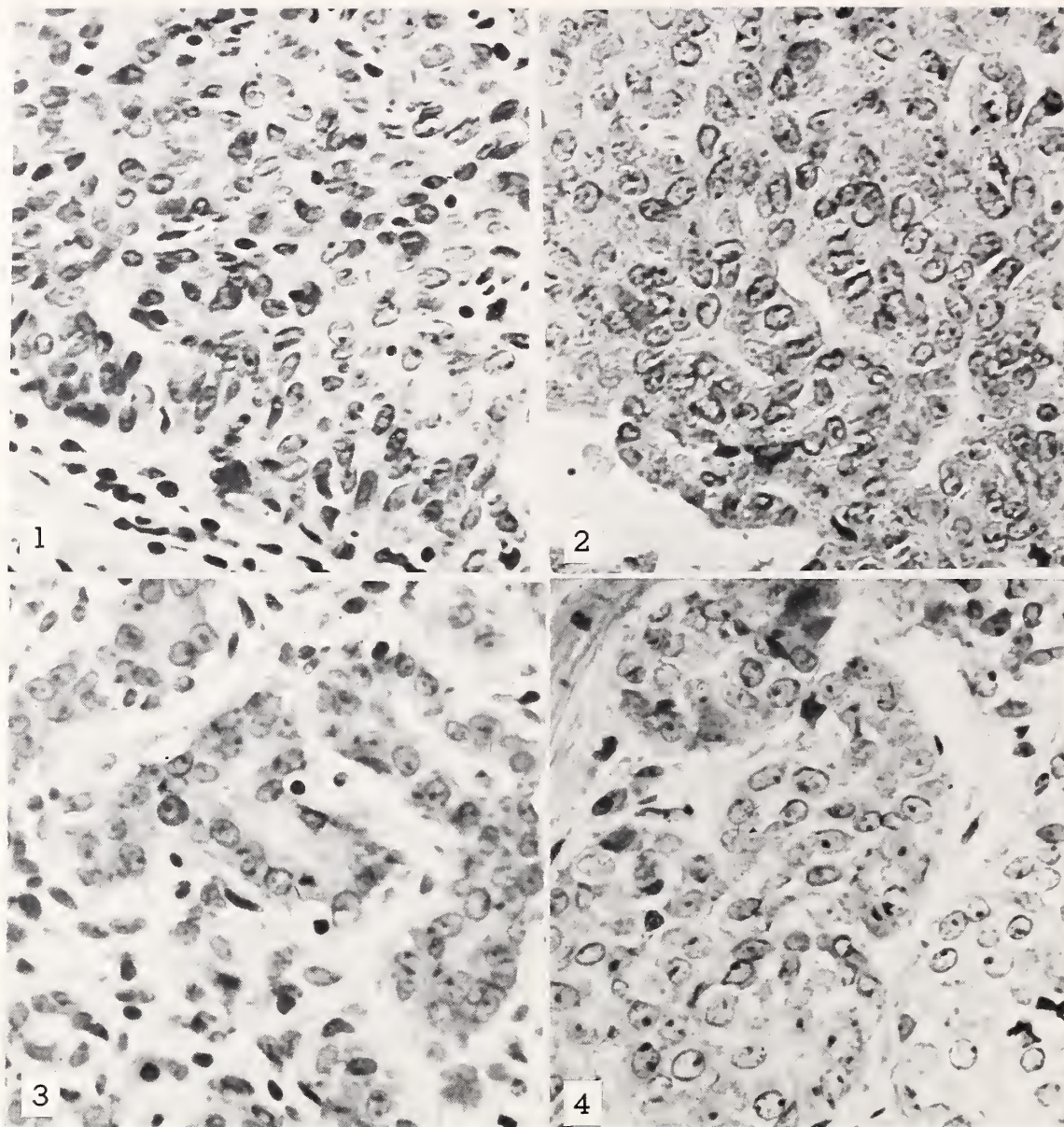


FIGURE 1.—Intraductal papillary hyperplasia ( $B_2$ ). Note scattered lymphoid cell nuclei surrounded by halo within papillary and lining epithelium.

FIGURE 2.—Intraductal atypical ( $B_4$ ) papilloma. Note absence of lymphocytes.

FIGURE 3.—Lobular hyperplasia ( $C_2D_2$ ). Note lymphocyte nuclei with surrounding halo within epithelium.

FIGURE 4.—Lobular atypia ( $C_4D_4$ ). Note absence of lymphocytes among atypical epithelial cells.

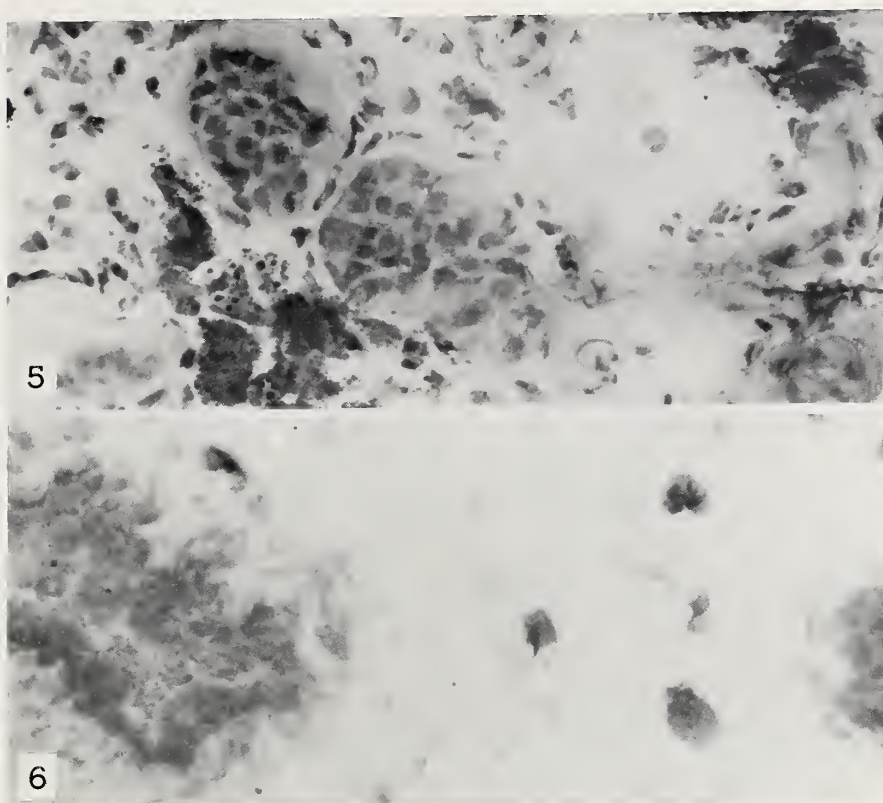


FIGURE 5.—Mast cells around duct atypia ( $C_3$ ); case 1524-72. Note close approximation of large mast cells to epithelium and enlarged granules. Polychrome methylene blue.

FIGURE 6.—Scattered mast cells in region of control ducts ( $B_1$ ); case 1524-72. Note difference in cell size, proximity to epithelial cells, and size of granules from those in figure 5.

of frankly invasive cancers (24). In a current study of mast cells, I have observed concentrations of markedly hypertrophied mast cells with enlarged granules close to duct atypia grade 3 and 4 in breasts with and without microinvasive cancer. As shown in figures 5 and 6, no such mast cell focalization or hypertrophy was seen in association with the control ducts or invasive cancer cells in the same breast. Nor were such mast cell concentrations seen in tissues from breasts lacking PCM or having widely infiltrating carcinoma. While the biologic significance of these observations is still obscure, the findings suggest that the earliest changes in epithelial-stromal cell relationships during carcinogenesis may not be immunologic in the conventional sense.

#### *Lymphoid Cell Responses to PCM*

As indicated above, 2 types of lymphoid cell responses were associated with invasive breast cancer: PVI and LI. As depicted in figures 7-10, analogous responses could also occur around foci of PCM. Here too, the PVI response was seen more frequently than the periductal lymphoid cell infiltration (PDLI) response. The PDLI response was usually limited to *in situ* carcinomatous changes (grade 5) characterized by anaplastic nuclei. As shown in figure 9, the PDLI response was commonly associated with a dense metachromatic fibrous reaction between the duct cells and lymphoid cells.

Local L-RE responses to PCM were seen most



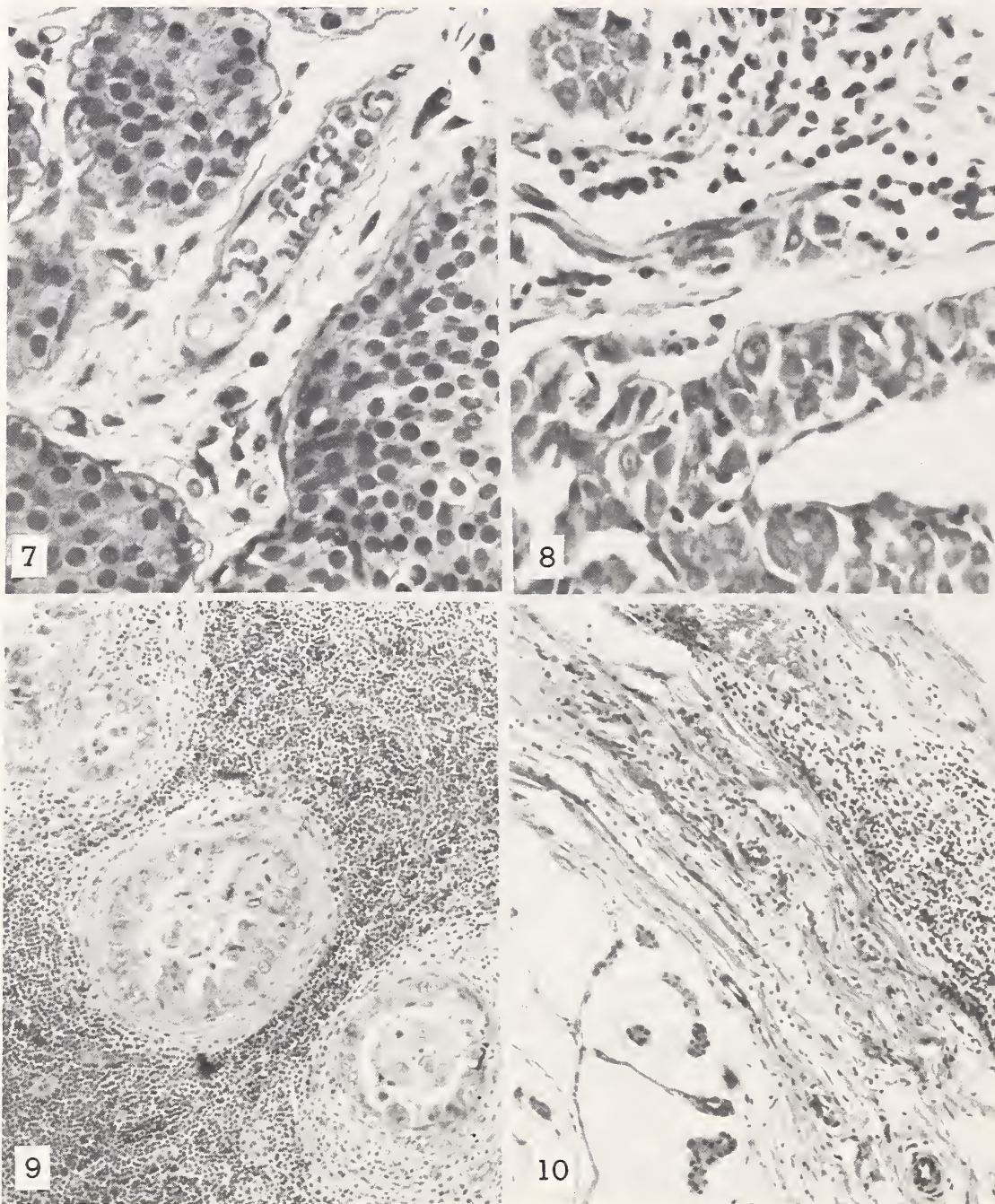


FIGURE 7.—Lobular atypic ( $C_3D_3$ ). No ILA or PVI response; case 4199-58.

FIGURE 8.—So-called lobular *in situ* carcinoma ( $B_5C_5D_5$ ). Note PVI response and lack of ILA; case 3174-71.

FIGURE 9.—*In situ* duct carcinoma ( $A_5B_5$ ). Despite intense PDLI, ILA are minimal; case 5190-70.

FIGURE 10.—Mucinous carcinoma of breast. Note intense PVI reaction at advancing edge of tumor; case MMB72-1.



TABLE 2.—Local L-RE response (PVI and/or PDLI) to PCM with and without associated invasive breast cancer

Duct grade	Without invasive cancer		With invasive cancer	
	Number	Percent positive	Number	Percent positive
3	21	19	15	20
4	12	50	11	18
5	31	84	105	56

often in grade 5 lesions. However, as indicated in table 2, similar reactions could also be seen in grade 4 and even grade 3 lesions. Foci of PCM in breasts with invasive cancer could also have associated L-RE responses. However, the relative frequency of such reactions tended to be somewhat lower in the latter group as compared to PCM without invasive cancer. Breasts having both *in situ* and invasive cancer provided a natural experiment for the comparison of the cellular responses to the *in situ* and invasive cells. As shown in table 3, there was a strong correlation between the response to *in situ* and invasive cancers in the same breast. These data were at least consistent with the thesis that *in situ* and invasive breast cancer cells have similar antigenicity and provoke cross-reactive cellular hypersensitivity responses.

#### Lymph Node Responses

Black and Chabon previously reported that axillary lymph nodes draining *in situ* carcinoma exhibited the same range of reactive and degenerative changes as were seen in lymph nodes draining invasive breast cancer (20, 23). Although the qualitative changes were similar, the frequency of the prognostically favorable SH response was greater in patients with *in situ* as compared to invasive cancer; *viz.*, moderate to marked SH responses were found in approximately 70% of patients with *in situ*

TABLE 3.—L-RE response to foci of PCM in cancerous breasts in relation to L-RE response to invasive cancer

Response to invasive cancer	Number	Response to PCM	
		Negative (%)	Positive (%)
Negative	40	35 (88)	5 (12)
Positive	41	4 (10)	37 (90)

carcinoma, in contrast to 50% of patients with Stage I invasive cancer and 30% of patients with unselected invasive breast cancers. The increased incidence of SH reactivity in patients with *in situ* cancer is in keeping with the increased incidence of PVI responses to *in situ* as compared with invasive cancers. Thus the cellular responses to PCM both locally and in the regional lymph nodes are consistent with the thesis that immunogenic changes are present in the preinvasive stage of breast cancer. However, the lymph nodes draining *in situ* cancers may show degenerative as well as reactive changes; thus, even in the earliest phases, breast cancer may produce diametrically opposite effects on the L-RE system, *viz.*, prognostically favorable and degenerative.

TABLE 4.—Skin window and tissue reactivity to breast cancer by stage

Stage	Positive/total (%)	
	Skin window	PVI/LI
<i>In situ</i> , microinvasive	10/13 (77)	10/13 (77)
Stage I	6/18 (33)	10/18 (56)
Stage II	7/26 (27)	11/26 (42)
Recurrent	1/6 (17)	2/6 (33)

#### Skin Window Responses

Further evidence of the immunogenic properties of the early phases of mammary carcinoma was provided by the cellular responses seen in skin window preparations. This procedure allows for direct assessment of the response of circulating lymphoid cells against selected samples of mammary epithelium. It also allows for repeated measurements at successive time periods. As shown in table 4, positive skin window responses to autologous cancer tissue were particularly frequent in patients with *in situ* or microinvasive breast cancer. With progressive stages of the disease, there was a decreasing frequency of skin window responsiveness. A similar relationship exists between stage and the local lymphoid cell responses. Prognostically favorable L-RE reactivity in the primary lesion, lymph nodes, and on skin window testing is the rule in patients with *in situ* cancers but is uncommon in patients with invasive breast cancer, particularly when the disease has progressed to the regional nodes and beyond.

TABLE 5.—Characteristics of breast cancer arising after removal of prior breast lesion

Prior lesion	Number postbiopsy cancer (% 5-year survival)	
	Number <i>in situ</i> / total cancers	Number invasive/ total cancers
None	(8%)	(50%)
Benign, grade <3	8/62 (13%)	28/54 (52%)
PCM	16/59 (27%)	29/33 (88%)
Invasive cancer	2/24 (8%)	5/15 (31%)

Considered *in toto*, the data strongly suggest that immunogenic changes occur *pari passu* with malignant transformation of mammary duct cells. They also suggest that prognostically favorable immunologic responses to PCM may cross-react with other breast cancers, in the *in situ* or invasive stage. If the latter presumption is true, then one would expect that breast cancer developing subsequent to the resection of PCM would face the handicap of pre-existing cross-reacting cellular hypersensitivity. Accordingly, such second cancers should be characterized by unusually favorable stage and survival characteristics. This natural experiment in tumor immunology was examined by Black, Cutler, and Barclay (25). The salient features derived from their study are presented in table 5. Patients with cancers who were previously treated for PCM had an unusually favorable prognosis in regard to stage and survival. Of 59 such cancers, 16 (27%) were *in situ*. This proportion of *in situ* cancers was some 3 times greater than would be expected in unselected series of breast cancers or among second cancers following mastectomy for invasive breast cancer. It was also twice as frequent as *in situ* cancers following biopsy of nonatypical breast lesions. Further evidence of a distinctively favorable prognosis for patients with cancers arising after removal of PCM was found in the percent 5-year postoperative survival of patients with invasive breast cancers: 88% versus 52% for invasive cancers arising after atypical and nonatypical breast lesions, respectively.

## DISCUSSION

The reality of immunologic responses to autologous cancer in man and experimental animals has received increasing support within the past

decade (26). It now seems reasonably well established that cancer cells possess distinctive antigenic characteristics and that prognostically favorable immunologic responses are of the cellular (delayed) hypersensitivity variety. In fact, data suggest that cancers provoke cellular hypersensitivity directed against antigens common to most, if not all, similar types of cancer: breast, neuroblastoma, melanoma, sarcoma, etc. (27-29).

The available data indicate that *in situ* carcinomas provoke similar types of L-RE responses as those provoked by invasive breast cancers. In both types of lesions the cellular characteristics of prognostically favorable L-RE responses in the primary tumor, the regional lymph nodes, and on skin testing support the thesis that cancer cells are immunogenic and provoke a cellular (delayed) type of hypersensitivity. In short, antigenic changes appear to accompany the earliest recognizable phase of malignant change. Of equal importance are data suggesting that the hypersensitivity against PCM cross-reacts with other breast cancers. Such cross-reactivity is a basic requirement for the ultimate development of immunotherapy and immunoprophylaxis.

While the present report emphasizes the frequent occurrence of cellular hypersensitivity in early breast cancer, it should not be overlooked that such reactivity becomes less common with progressively extensive disease. An understanding of the mechanisms involved in such changes would be of practical as well as conceptual interest. The Hellströms suggested that progressive growth of cancer is related to the development of blocking antibodies which subvert the cellular hypersensitivity (30). One might expect that the active production of circulating blocking antibodies would be correlated with a hyperimmune type of lymph node pattern, *viz.*, prominent secondary follicles and plasma cells. However, no such correlation is seen in the lymph nodes of patients with advanced breast or gastric cancer. Nor do the lymph nodes appear indifferent to the presence of such cancers at any stage of the disease from *in situ* to widely disseminated. On the contrary, advancing disease tends to be correlated with increasing frequency of degenerative changes, which include degenerative SH, sinus fibrosis, and lymphoid pulp atrophy (5, 31). The reason for the apparent discrepancy between lymph node structure and *in vitro* measurements is not now apparent. Correlative studies of



*in vitro* response and L-RE reactivity in individual patients might be most informative.

If, as seems probable, immunologic reactions limit the aggressive potential of breast cancer cells, it is pertinent to question the *modus operandi*. Experimental *in vitro* studies on lymphotoxins, rosette formation, and colony inhibition have led to the tacit belief that the lymphocyte functions *in vivo* as the "killer" of cancer cells. Such killing is presumed to require direct contact with the target cell. This interpretation appears consistent with the prognostically favorable LI reaction. However, LI is an uncommon type of response even in minimally progressive breast cancers. It could hardly qualify as the structural representation of anticancer immunity in most breast cancer patients. On the other hand, the more frequently observed prognostically favorable L-RE responses, *viz.*, SH and PVI, are not characterized by prominent lymphocyte-cancer cell contacts. These findings suggest that either lymphocytes can act at a distance or some, as yet unknown, mechanism is involved. The important point is that the biologic realities of human breast cancer are found in patients with breast cancer. Consistency with *in vivo* events should be a minimum requirement of hypotheses regarding the biologic behavior of human breast cancer.

While much remains to be learned of the development and progression of breast carcinoma, the available data amply confirm the participation of immunologic phenomena in the tumor-host relationship. The data also support the 1950 suggestion of Black, Bolker, and Kleiner that "...at least in its initial stages, malignant neoplasia represents a delicate balance between the local growth and the milieu of the host..." (1). PCM provides uniquely favorable material for examining the nature and components of this balance.

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# Immunologic Detection of C-Type RNA Viral Reverse Transcriptases<sup>1, 2</sup>

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**SUMMARY**—Discovery of a reverse transcriptase in RNA tumor viruses and subsequent development of antibodies directed against this enzyme led to the development of sensitive biochemical methods for C-type virus detection. Antibody directed against the murine leukemia viral reverse transcriptase markedly inhibited that enzyme but did not affect two major polymerase activities found in normal mouse cells. With antibodies against avian, feline, and murine C-type enzymes, partial cross-reactivity was demonstrated between C-type polymerases of mammalian species including the rat, mouse, cat, and hamster. None of the antibodies inhibited reverse transcriptase activity of non-C-type viruses, including mouse mammary tumor virus, visna, and foamy virus. Also, three recent isolates of primate C-type viruses were not inhibited by antibodies against either murine or feline C-type enzymes. These studies indicate that at least certain mammalian C-type reverse transcriptases contain both species-specific and interspecies antigenic determinants. When used in combination with sensitive biochemical methods for measurement of reverse transcriptase activity, the antibodies are demonstrated to be useful in identifying very low levels of virus production.—*Natl Cancer Inst Monogr* 35: 83–87, 1972.

IN 1970, Baltimore *et al.* (1) and Temin and Mizutani (2) discovered that RNA tumor viruses contain an enzyme synthesizing DNA from a viral RNA template. Further studies showed that the RNA C-type viruses of all animal species from which isolates have been obtained (3–5) and certain other RNA containing viruses, such as visna (4, 6, 7), mouse mammary tumor virus (3, 4), monkey mammary tumor virus (4), and syncytium-forming vir-

ruses (8), contain this unique enzyme. The enzyme reaction for detection of viral polymerase can be greatly increased in sensitivity by the use of synthetic templates (9, 10) and the appropriate divalent cations (11). Immunologic methods enabling identification of the source of reverse transcriptase activity have recently been developed. The present paper reviews findings which led to the development of antisera to viral polymerases and some of the applications of these antisera to problems related to viral oncology.

## **Antibody to Murine C-Type Viral Reverse Transcriptase in Sera of Animals With Tumors Induced by Murine Sarcoma Virus (MSV)**

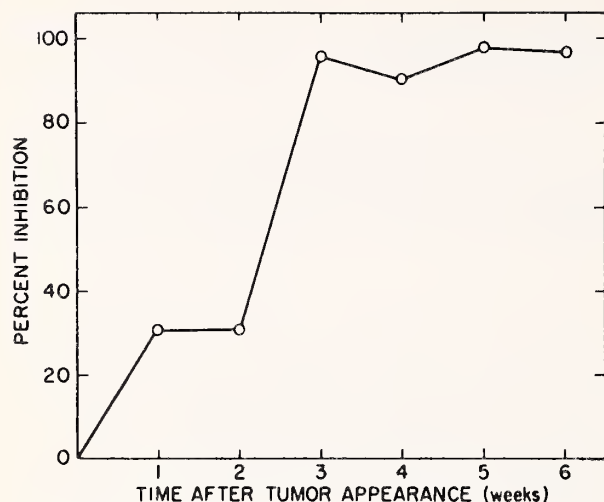
In 1970, Gerwin *et al.* (12) observed that sera of rats bearing transplantable, MSV-induced tumors contained an inhibitor of the enzyme activity of

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TEXT-FIGURE 1.—Time course of appearance of serum inhibitor. Sera, obtained at weekly intervals after inoculation of a Fischer rat with MSV tumor (each serum containing  $25 \pm 5 \mu\text{g}$  protein), were added to reaction mixtures incubated at  $37^\circ\text{C}$  for 60 minutes and contained in 0.05 ml:0.02 M Tris·HCl, pH 7.8; 2 mM dithiothreitol; 0.04 M KCl;  $1 \times 10^{-3}$  [ $^3\text{H}$ -methyl]thymidine triphosphate (5000 cpm/pmole); 1 mM manganese acetate; 0.01% Triton X-100; 0.006  $A_{260}$  polyriboadenylic·polyuridylic acid (A.U); and 1  $\mu\text{g}$  Rauscher MuLV. The incorporation in the absence of rat serum was 50,000 cpm.

disrupted virions of murine leukemia virus (MuLV). Further studies revealed that this serum inhibitor had a time-dependent development in the animals. As shown in text-figure 1, inhibitory activity was detected as early as 1–2 weeks and reached maximal levels by 3 weeks after tumor inoculation. This pattern suggested an antibody response. As summarized in table 1, the inhibitor had the following physiochemical properties: It was resistant to dialysis, precipitable in 50% ammonium sulfate, and cochromatographed with IgG on Sephadex G-200. These results, along with the finding that the serum inhibitor specifically bound to viral enzyme and not to template, led to

TABLE 1.—Characteristics of serum inhibitor

- 1) Not dialyzable.
- 2) Precipitated by 50% ammonium sulfate.
- 3) Eluted at 0.01 M phosphate from DEAE\*-cellulose.
- 4) Having an  $E_{280}/E_{260}$  ratio of 1.48 after DEAE-cellulose chromatography.
- 5) Included in Sephadex G-200; peak of inhibitory activity corresponded with IgG. Molecular weight was about 160,000.

\* DEAE = diethylaminoethyl.

the conclusion that the inhibitor was an antibody to the MuLV reverse transcriptase (13).

### Development of Antibodies to Partially Purified Viral Reverse Transcriptase

Viral reverse transcriptase was partially purified by use of a 2-step procedure: Sephadex G-100 followed by phosphocellulose chromatography (14). This method resulted in at least a 100-fold purification of the enzyme from virions. To obtain antisera directed against the reverse transcriptase itself, minute quantities of purified enzyme were used to immunize rabbits. Antibody production was first demonstrated against the enzyme obtained

TABLE 2.—Cross-reactions between DNA polymerases of different viruses and antipolymerase sera

Viral polymerases inhibited by:		
Anti-pol (MuLV)*	Anti-pol (AvLV)†	Neither
MuLV	SR-RSV	Viper
RaLV	B-RSV	Murine mammary tumor virus
	RAV-1	Visna
HaLV	RAV-2	MP-MV‡
FeLV	AMV	Simian "foamy"

\* MuLV = murine leukemia virus; RaLV = rat leukemia virus; HaLV = hamster leukemia virus; and FeLV = feline leukemia virus.  
 † AvLV = avian leukemia virus; SR-RSV = Schmidt-Ruppin strain of Rous sarcoma virus; B-RSV = Bryan strain of Rous sarcoma virus; RAV-1 and -2 = Rous-associated viruses; and AMV = avian myeloblastosis virus.

‡ Mason-Pfizer monkey virus.

from the Rauscher strain of MuLV (13, 15). Subsequently, antisera have also been prepared against the reverse transcriptases of avian (15) and feline (5) C-type viruses. The activity of each antiserum was shown to be that of an IgG serum protein specifically inactivating viral enzyme but not template.

### Inhibition of Different Template-Stimulated Reactions by Antipolymerase Sera (Anti-pol)

The synthetic template, polyriboadenylic · oligodeoxythymidylic acid, because of its great sensitivity, is commonly used to detect reverse transcriptase activity. Rabbit antiserum against the MuLV reverse transcriptase—antipol (MuLV)—markedly inhibited the activity of this polymer-stimulated reaction. Parks *et al.* (15) showed that



anti-pol (MuLV) was equally effective against other templates utilized by the polymerase, including viral RNA and "activated" DNA. The results of these studies support other results (14, 16) suggesting that both the RNA-dependent and DNA-dependent DNA polymerase activities are associated with a single enzyme.

### Immunologic Relatedness of Viral Reverse Transcriptases of Different Species

Antisera to reverse transcriptases of RNA tumor viruses have been useful in studying the antigenic relatedness of different species of viral enzymes. As shown in table 2, rabbit anti-pol (MuLV) inhibited the activity of C-type rat, hamster, cat, and mouse reverse transcriptases. Thus there is an interspecies antigenic relatedness of certain mammalian viral polymerases. In contrast, anti-pol (MuLV) did not inhibit the activity of polymerases of avian C-type viruses or reverse transcriptases of several non-C-type viruses. As shown also in table 2, rabbit anti-pol (MuLV) markedly inhibited the polymerase activity of all strains of avian C-type virus but was ineffective against enzymes of mammalian C-type viruses. The last findings demonstrate the immunologic similarity of viral polymerases of different strains of avian C-type viruses. The avian C-type enzymes appeared, however, to be antigenically unrelated to enzymes of mammalian C-type viruses (15).

Table 3 shows the results of enzyme inhibition studies in which antisera to 2 mammalian C-type viruses, anti-pol (MuLV) and anti-pol (FeLV), were tested against several species of viral reverse transcriptases. Each antiserum was used at a concentration which resulted in >90% reduction of the activity of its homologous enzyme. Inhibition of the homologous polymerase was in each case greater than that seen in the reciprocal cross. Anti-pol (FeLV) was more effective against FeLV than against MuLV polymerase, whereas anti-pol (MuLV) more strongly inhibited MuLV enzyme activity. While both antisera clearly inhibited the activity of hamster and rat polymerases, they were less effective than against their homologous enzymes.

Recently, C-type viruses were isolated from tumors of a woolly monkey (17, 18) and a gibbon ape (19). Another isolate was obtained from a human tumor, RD-114, after passage through an cat em-

TABLE 3.—Inhibition of C-type viral polymerases by anti-pol (MuLV) and anti-pol (FeLV)\*

Virus source	Percent inhibited by	
	Anti-pol (MuLV)	Anti-pol (FeLV)
Mouse	>95	50
Cat	60	>90
Rat	60	40
Hamster	40	40
Woolly monkey	<5	<5
Gibbon ape	<10	<10
RD-114 (a human tumor)	<5	<5

\* See (5) for experimental details.

bryo (20). These viruses have many characteristics of mammalian C-type viruses, yet lack the major group-specific (gs) antigen of any previously isolated species of C-type virus. The reverse transcriptase of each virus was not significantly inhibited by either anti-pol (MuLV) or anti-pol (FeLV) (table 3). These results imply that the reverse transcriptases of higher mammalian C-type viruses are immunologically less closely related to those of the mouse or cat (5). These studies demonstrate the need for antisera directed against the polymerases of primate viruses. Such antisera eventually may help in the identification of viruses of potential human origin.

### Other Applications of Anti-pol Sera

Anti-pol (MuLV) has been useful in the study of the antigenic relationship between the viral reverse transcriptase and certain cellular enzymes which can utilize synthetic RNA-DNA and RNA-RNA templates (27). Ross *et al.* (14) demonstrated, in the mouse model system, that 2 cellular polymerases could be isolated from normal BALB/3T3 cells. A third enzyme activity could be detected in BALB/3T3 cells chronically infected with MuLV. The additional peak of polymerase activity migrated differently on both Sephadex G-100 and phosphocellulose than either of the 2 normal cellular enzymes. Anti-pol (MuLV) markedly inhibited this third peak of enzyme activity. In contrast, the antiserum did not inhibit either normal cellular polymerase, indicating the lack of antigenic relatedness between these enzymes and the C-type murine viral reverse transcriptase. In both normal and tumor cells of human origin examined, 2 enzyme activities were demonstrated. These activities

resembled in many physical properties and template preferences those obtained from normal mouse cells (14). A third polymerase activity similar to that of the murine transcriptase was not demonstrated. The eventual development of antisera that inhibit reverse transcriptases of primate C-type viruses may make it possible to determine whether polymerase activities obtained from human cells are viral in origin.

Another important application of anti-pol serum has been in identifying virus with little or no biological activity as determined by standard techniques. Recent studies, for example, showed that C-type RNA viruses could be activated by treatment of AKR mouse-embryo cell lines with chemicals such as bromodeoxyuridine (22). Treatment of BALB/c clonal lines with these same chemicals did not yield detectable levels of infectious virus, but low levels of reverse transcriptase activity could be demonstrated in concentrated culture fluids of treated cells (23). The ability to inhibit this enzyme activity with anti-pol (MuLV) made it possible to conclusively demonstrate the inducibility of C-type virus from a large number of clonal lines of embryo cells of a mouse strain having low-leukemia incidence.

## CONCLUSIONS

The present report describes the development of antisera that specifically inhibit viral reverse transcriptases. The usefulness of such sera for distinguishing C-type viral from cellular enzymes and other viruses containing reverse transcriptases, as well as for the detection of virus production where biologic assays are unavailable, has been demonstrated. This immunologic approach may eventually be helpful in the identification of C-type viruses of primate and potential human origin.

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## Summary: Antigens in Preneoplastic Tissue During Tumorigenesis<sup>1</sup>

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IN SUMMING up Session 2, I shall re-emphasize a number of salient points made by the speakers, add a few related comments, and primarily, set our discussion within the framework of the central question underlying the whole field of the immunology of carcinogenesis today: What is the relevance of immunologic surveillance in nature?

If immunologic surveillance against tumor cells does have a major function in phylogenetic adaptation to the challenge of neoplasia, then it must already come into play at the initial confrontation between a newly arisen population of aberrant cells and the immune mechanism of the host, early after the appearance of the new hostile clones. Thus, in those instances in which clearly defined *preneoplastic* cells appear as an intermediary stage between normalcy and frank neoplasia, the first battleground between the variant cells and the host is at the preneoplastic level. Although well-characterized preneoplastic cells have been described so far in only a few tumor systems, they may well occur in many, and perhaps all, processes of neoplastic transformation, even if sometimes only transiently.<sup>2</sup>

I shall immediately focus on the consideration of which opportunities may uniquely allow immunologic surveillance to express itself positively against cells at the *preneoplastic* stage, and what, on the

other side of the coin, can be said for any special ability of *preneoplastic* cells to escape immunologic surveillance. In other words, we must center our discussion of the capacities of immunologic surveillance and the means of escaping from it at the preneoplastic level, where the question is posed in nature, and not at the stage of full neoplasia, where we are probably dealing with events already beyond the critical interaction.

The fundamental assumption on which this discussion is based must be, of course, that preneoplastic cells are already distinguished by an antigenicity sufficiently deviant in kind or degree from normal to evoke immunologic reactivity (and preferably cross-reactive at least partially with that of more fully neoplastic cells in the chain of a given neoplastic progression). A number of published reports dealing both with virus-induced and other neoplasms (1-4) and some of the information presented here by our speakers suggest strongly that this is, indeed, the case, at least for the presently known models of distinctive preneoplasia.

Let us first consider some of the factors which make immunologic surveillance seem more likely to succeed against preneoplastic than neoplastic variants:

1) There is the obvious consideration that, early after the appearance of cells with preneoplastic properties and with accompanying antigenic changes of a magnitude which make autochthonous immunologic recognition possible, the immunologic apparatus is not yet faced with an overwhelming burden; the number of "alien" cells to be destroyed is still well within the bounds of feasible homograft reactivity as we know it in many experimental systems. The chances for the induction of specific immunologic tolerance or paralysis, or merely of mechanical or physiologic inaccessibility of the aberrant cells to immunologic elements, are minimal. It is self-evident that most can be expected of the immune response when the challenge

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<sup>1</sup> Presented at Conference on Immunology of Carcinogenesis, held by the Oak Ridge National Laboratory at Gatlinburg, Tenn., May 8-11, 1972.

<sup>2</sup> It is not important for this discussion to go into the question of whether the term "preneoplastic," favored by K. B. DeOme, or "pre-malignant," employed by R. T. Prehn, is more appropriate. Both terms describe early stages in a progressive series of events ultimately leading to the characteristics of full neoplasia; both designate cells which, though no longer functioning as normal, still lack some properties of those at the terminal end of the spectrum of neoplastic change, including malignancy where invasiveness and metastatic spread are among the attributes of the progression in its final manifestation.

it faces is very limited, *i.e.*, a small number of antigenically foreign cells that have not yet had the time to form morphologic aggregations with boundaries which might be exceptionally impervious.

2) During the early period after the transformation of a single (or a number of?) cell(s) to preneoplasia, the probability that extensive metastatic seeding has already taken place is at least statistically more limited than when a large primary tumor mass is already in existence.

Metastatic colonies of many primary cancers ultimately appear, as we know, diffusely throughout the body, but it may be that metastatically disseminated cells develop initially in immunologically privileged sites, as a kind of holding operation. They may, perhaps, develop only slowly at first in such intermediary localities, and may remain undetected until a fall in the immunologic capacity of the host or a crucial alteration in immunoresistance properties of some of their subclones permits escape from surveillance—and by that time, their number both in the initial and in secondary sites of metastatic localization may be such as to overcome remaining immunologic defenses, even in nonprivileged tissues and organs. Thus, the longer such a subterranean process of occult dissemination continues, the greater become the dangers that one or even an explosive number of foci will suddenly be enabled to develop progressively. The decisive attempts by the host for a categorically successful abortion of metastasis may thus have to be mounted long before any satellite colonies make a gross appearance. These attempts may be most effective early, at the moment when the potentially metastatic cells break off the initial and perhaps still largely preneoplastic growth and begin to wander, and before they are ensconced in a protected holding site.

3) Shortly after the beginning of an eventually neoplastic process, there is still only a small likelihood of secondary mutation, and/or of the selection of any such mutants, to reduced or nonoperative levels of (accessible) tumor-associated antigenicity on the cell membrane or to unique properties of refractoriness to immunologic attack. Such mutational and selective processes occur; but if one puts aside inherent differences from tumor to tumor in the frequency and efficiency of such occurrences, their probability must increase in parallel with the size of the variant population.

4) To speculate, I would like to throw out a

suggestion based on Jerne's concepts of the origin of antibody diversity (5): There may be an optimum range of immunogenicity of tissue-related antigens and perhaps these antigens are most effectively immunogenic when they are neither too closely related to, nor too far removed from, the chemical or biochemical structure and steric conformation of normal histocompatibility determinants. Such an optimal range could conceivably be multiphasic, with subranges of maximal immunogenicities lying at different and perhaps overlapping levels of magnitude for antigenicities divergent from self, from the strain, from the species, and from other biologic categories. Thus, an allogeneic antigen might be most effective when located at a certain distance from self and might be either less or more immunogenic than a species xenogeneic one, whose optimum immunogenic strength would, in turn, be at a fixed locus within the range of antigenic variation of that species from the self-antigenicity of the host in question. Highly speculative as this consideration is, there argues for at least some elements of it the observation that, in some experimental systems, the efficiency of lymphocyte transformation in the presence of more closely related allogeneic target cells is much greater than when the target cells are far removed, or even xenogeneic. The recognition of the alien could, in other words, depend not only on the degree of foreignness but also on the degree of kinship.

If this suggestion is at all valid, then perhaps, at a crucial, early stage in a neoplastic event, the population of aberrant clones might be maximally immunogenic! Later, with the changes to full neoplasia, secondary and tertiary morphologic changes giving rise to new antigenicities might remove the population beyond the optimum point of immunogenicity for the antigenic category. This might be more true for cellular immune phenomena, with their occasional demands for greater specificity (*i.e.*, greater proximity to self-configurations?) than for humoral antibody responses, which can be strong though directed at very isolated molecular determinants. Thus, there may occur "antigenic progressions" side by side with the progression of other changes during neoplasia, and the progressive or cumulative antigenic deviations might be one of the means of evasion of cellular immunologic surveillance.

Similarly, if it is true that anti-antibodies (*i.e.*,



idiotype antibodies) can attack the host's cytotoxic antibodies or effector cells with analogous new receptors (6, 7), and thereby damage the efficiency of immunologic surveillance, this could be considered as increasingly likely with time and change after the initial, focal preneoplastic event: As the antigens associated with more and more flagrantly neoplastic cells become "stranger" and more deviant from self, so may the immune elements directed against them and, in turn, their own capacity to incite immunologic attack.

5) It may be worthwhile to mention another, though also problematic reason for assuming the greater opportunity of efficacious immunologic surveillance against preneoplastic than against wholly neoplastic targets. Mr. Ben Sasson in our Department and investigators in several other laboratories, including Dr. I. P. Witz from Tel Aviv University, have recently observed that at least some neoplastic cells in tissue culture shed into the medium rapidly and continuously significant quantities of membrane fragments carrying tumor-associated antigenicity (8). If such peeling off also occurs *in vivo*, one can imagine the creation of gradients of tumor-associated antigens surrounding foci of neoplastic cells. Specific immune elements, whether cellular or humoral, would have to transverse such gradients to attack their targets, and such gradient formation may thus represent a major defense mechanism by neoplastic variants. Although this point is still far from clear, the shedding of antigenic membrane components may increase with the advance to full neoplasia, and preneoplastic cells may still be unable to express this defense fully.

Some other specific mechanisms of escape associated with preneoplasia can be envisaged, but the several suggestions here hazarded are sufficient to make the point: Immunologic surveillance can be imagined to be especially effective, for various reasons, at the earliest stages of the transformations leading to full neoplasia.

On the other hand, circumstances can also be postulated for the distinctive facility of preneoplastic cells to *evade* immunologic monitoring:

1) I should like to refer to the observations of Dr. Slemmer that, during progression to full malignancy, preneoplastic (Dr. Slemmer would term them "premalignant") cells "...were found to require continued association with normal cells for survival as well as for optimal growth and progres-

sion to malignancy," and that "...progression to malignancy by the neoplastic cell component of a mosaic premalignant tissue resulted in the loss of the association with and dependence on normal parenchymal cell components." Dr. Slemmer further noted that "...morphologically normal mammary cell components, frequently containing small numbers of premalignant cells hidden in their structures, were left behind" after the immune rejection of 3-methylcholanthrene-induced premalignant tissues by immune lymphocytes. Thus, the dependence of preneoplastic cells on intimate spatial association with wholly normal ones may also provide the variants with a kind of mechanical shelter in which they can survive, as isolated and dangerous islands, a flood of immunologic hostility from their host.

2) There are grounds for believing that frequently prominent among the different categories of tumor-associated antigens are organ-specific ones, expressed on neoplastic variants of cell types on which they normally do not appear, or on which they are found in much smaller concentrations when the cells are normal. Organ-specific antigens may have a special capacity to evoke the formation of free antibodies with enhancing activities (9), or at least fail to provoke the formation of cytotoxic immune factors; this may, indeed, be a major mechanism of self-tolerance (10). Could it not be, then, that clones of preneoplastic and neoplastic cells which tend especially to form organ-specific antigens on their cell surface will thereby be endowed with survival advantage? Such a pre-emption by a population of pathogenic cells of a normal homeostatic control mechanism would certainly not be without precedent. If preneoplastic cells, then, are more usually or more pronouncedly distinguished by the expression of certain organ-specific immunogens than are fully neoplastic ones—and if one considers the common loss of normal antigens by neoplastic cells, especially anaplastic ones, this eventuality would not seem to be too far-fetched—a better prospect for the survival of the preneoplastic variants in an immunologically ambivalent environment could be postulated.

3) It is also reasonable to assume that the effective tumor-associated antigenicity in preneoplasia may often be intrinsically marginal. The number of aberrant cells in the early phases of a neoplastic transformation is small, and the total antigenic pool would be correspondingly restricted. More-

over, departure from normal histocompatibility antigenicity may be insufficient in the early period to enable a potent immune reaction. Accordingly, the host may permit preneoplastic variants to multiply and spread for some time until it can offer an appreciable immunologic response—and then the shift to specific tolerance or paralysis may occur so precipitously that sufficient numbers of the pathogenetic variants may survive to initiate progressive disease beyond the reach of immunologic surveillance.

These considerations also bring up the question of whether interference in the equilibrium between preneoplastic cells and their hosts by nonspecific modulators of immunologic responsiveness can strengthen host defenses. This is discussed elsewhere in this monograph (11), and here I shall only point out that studies conducted with Dr. Howard Bern some years ago at the University of California at Berkeley did, in fact, indicate that treatment with the methanol-extraction residue fraction of tubercle bacilli markedly impedes preneoplastic development of the mammary parenchyma of mice (12). Thus, even when the immunologic apparatus is not by itself capable of inhibiting preneoplastic growth and subsequent change to full neoplasia, extrinsic modulators of resistance may elevate responsiveness sufficiently to make this possible.

Such intervention could conceivably take on both a prophylactic and a therapeutic form. One possibility was presented in the results reported at this Conference by Dr. Aaronson—that the presence of (unusually high?) levels of an oncogenic virus in tissues may be diagnosed by detection of enzymes specifically associated with the agent. Alternatively, antigens characteristically associated (perhaps even only quantitatively) with the neoplastic and/or preneoplastic state might be detectable immunologically in body fluids. With such presumptive evidence at hand of an incipient neoplastic process, or of a heightened risk that such a process may occur, treatment with nonspecific stimulators of resistance and of immunologic responsiveness might be indicated—when such manipulation might be most likely to be maximally effective. Neither is it impossible to conceive of the feasibility of such nonspecific immunologic intervention in individuals who show, on routine periodic examination of their immunologic profile, a marked decline in cellular and IgM capacity, and who by

reason of age, genetic background, or environmental exposure may be judged as facing an above-normal risk of developing malignant disease.

In summary, I have alluded to several mechanisms that may provide the immunologic apparatus with unique opportunities to hinder neoplastic development at the stage of preneoplasia, and to other mechanisms that may allow the preneoplastic cell to escape. It may well be that, precisely at the preneoplastic level, the struggle between variant cells with neoplastic potential and the host is fought most decisively; it may also well be that victory by the host at this stage accounts for the relative infrequency of progressive malignant disease in outbred populations.

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## GENERAL DISCUSSION

**M. A. Basombrio:** The application of 3-methylcholanthrene (MCA) to the skin produces some degree of hyperplasia before papillomas arise, and I would ask Dr. Lappé if he finds that skin with this hyperplasia, or before it develops papillomas, shows any sign of immunologic rejection.

**M. A. Lappé:** The initial work that uncovered the phenomenon of graft promotion addressed itself to that question. That is, first- and second-set grafts were made of MCA-treated skin to see if there was any evidence of immunologic rejection, focally or grossly over the graft as a whole. Unless the graft was treated with enough MCA to make it necrotic, there was no evidence of a second-set type of response to a second isograft after acceptance of an initial MCA-treated isograft. A very large series of experiments was also done to see if pretreatment with an initiated isograft affected the subsequent growth of papillomas on another isograft, and the results were negative.

**M. M. Black:** With regard to the system you described, I would like to call attention to a different type of cellular response, which suggests that there may be cellular changes occurring before what we conventionally think of as immunologic responses. I refer particularly to the work of W. L. Simpson a good number of years ago on the mast cell response that occurs in carcinogen-induced atypical hyperplasias. When the lesion becomes a true invasive cancer, these mast cells disappear.

We see the same thing in breast carcinoma. In very special cases which mimic the Simpson studies, many areas of tissue not quite what we would call a precancerous mastopathy contain gigantic hypertrophied mast cells. They are 4 times larger than normal, their granules are enlarged, and they cluster right up against the epithelial cells.

In the control areas of the same specimens just a few millimeters away, you do not see them. Nor are they seen in invasive areas. Also, in the lymph nodes with sinus histiocytosis reactions, you typically have increased numbers of mast cells.

I ask the audience: What is a mast cell, and what does it do?

**H. Schreiber:** Dr. Black, you showed that in patients from whom carcinoma *in situ* in the breast was removed, the 5-year survival after the occurrence of invasive carcinoma was better than that in patients from whom benign lesions were removed and invasive carcinoma developed afterward. Were the patients with the breast carcinoma *in situ* screened more carefully and more often than patients with benign lesions?

**Black:** I apologize for not clarifying the nature of the population. In the first place, you inferred that they were followed more closely and would therefore have a better prognosis. I wish that were really true, for this certainly is not the general clinical experience. If it were, we wouldn't be sitting here talking about breast cancer problems or having meetings about these problems. You would have a very simple solution. All of the lesions, the nonprecancerous

mastopathy and the precancerous mastopathy group, had been diagnosed in Saskatchewan as just plain benign. They were handled identically.

The differentiation into the 2 subcategories by the nature of the lesion was done by me under double-blind conditions without knowing what happened to the cases.

**A. K. Szakal:** With regard to Dr. Lappé's answer, that there is no evidence for rejection of carcinogen-pretreated isografts, my observations show that, on immunologically competent recipients, 7,12-dimethylbenz[*a*]anthracene (DMBA)-pretreated isografts never developed papillomas and were rejected with a decrease in mean graft survival time directly in proportion with the increasing number of DMBA treatments. Also, pretreated autografts on immunologically depressed (via DMBA) recipients developed papillomas and were chronically rejected with an increased mean survival time. You will see from my presentation some of the reasons I do not believe that these were due to carcinogen-induced necrotic changes in the graft.

**S. Collins:** Dr. Aaronson, will you comment on the credibility of reports on the presence of reverse transcriptase in normal cells, such as in phytohemagglutinin-stimulated lymphocytes, and also on the proposed role of reverse transcriptase in the normal functioning of immunologically reactive cells?

**S. A. Aaronson:** A number of studies have been concerned with the properties of polymerases obtained from mammalian cells; some of these enzymes have been termed reverse transcriptases. Our studies were carried out first in the mouse model system, in which, as I explained, immunologic reagents were available to help specify viral from normal cell enzymes. In normal mouse cells, we found 2 enzymic activities. We could classify neither as viral by any of several properties, such as template preferences, chromatographic migration patterns, or inhibition by antibody made against known C-type viral reverse transcriptase.

In all human tumor cells we have examined, or in all normal cells from humans we have examined to date, we have found 2 enzyme activities which resemble, in migration and template preferences, those of the normal mouse enzymes. These polymerases can be made to work with synthetic RNA or RNA-DNA templates. Anti-pol sera that recognize primate C-type viral enzymes may help in determining whether polymerase activity detected in human cells is viral in origin.

Regarding your second question on the possible role of a reverse transcriptase in immunologic recognition, such an enzyme has been suggested in this process as well as in memory and other biologic functions. No experimental evidence relates to these possibilities as yet, but these possibilities certainly provide exciting areas for much future work.

**S. Rosenberg:** Dr. Weiss, you and several other speakers have talked about premalignant, or preneoplastic, cells. What is a premalignant cell? Aren't you implying a particular mechanism of malignant transformation when you talk about a premalignant cell? Could not the transition to

malignancy be a one-hit type phenomenon, wherein the cell is normal and then becomes malignant with no intervening, meaningful stages? To help the discussion further, could you define what you mean by premalignancy?

**D. W. Weiss:** I would prefer having Dr. Slemmer define this. I discussed this issue in my Summary of this session.

**G. L. Slemmer:** My feeling on the difference between the Prehn and the DeOme terminologies is that the DeOme terminology derives, to a certain extent, from a tendency to equate neoplasia with malignancy. The terminologies of Drs. L. Foulds and R. T. Prehn consider the early lesions as benign neoplasms. As I understand your question, you are more interested in what is premalignancy—how do we know or why do we say a tissue is premalignant?

The premalignant tissues can be shown, by means of transplantation studies, to give rise to malignancy more often and in less time than transplants of adjacent normal tissues.

On this basis, we say they are premalignant.

**Rosenberg:** But they may already be malignant?

**Slemmer:** Malignant tissues have the capacity for malignant invasion and metastasis and will kill the host, whereas these premalignant tissues do not have those capacities. They are intermediate between normal tissues and malignant carcinomas.

**Weiss:** It seems to me—if I may just add one more point to this—that what we are dealing with here is an attempt to define precisely a very fluid situation. I view this whole issue as representing a spectrum of stages beginning with a totally normal cell—whatever that means—to a cell that, by any accepted criteria of metastatic ability, full invasiveness, etc., may be termed fully sarcomatous or carcinomatous. In any situation in which one attempts to give a static definition of a very dynamic condition, intrinsically labile and fluid, the definition is never wholly satisfactory.

**W. J. Martin:** Dr. Slemmer's study is relevant to the mechanism of graft rejection. In his study and in other studies using mosaic tissue, containing a mixture of syngeneic and alien tissues, one can find selective destruction of only the alien tissue with preservation of the syngeneic tissue within the transplant.

These studies suggest that *in vivo* graft rejection may be highly discriminative, destroying tumor but not normal tissue. This type of destruction is more consistent with the activity of specially reactive sensitized lymphocytes rather than of nonspecific, so-called angry macrophages. In your study, do you observe any destruction of normal tissue during the complete elimination of the apparently neoplastic tissue and vice versa?

**Slemmer:** That's an important point that I failed to make in my discussion. Though the syngeneic tissue is sometimes neoplastic, in the presence of an allograft-immune response, the neoplastic tissue is not rejected.

This suggests that the phenomenon of nonspecific cytotoxic effects studied in Dr. Rapp and Dr. Zbar's laboratory may not apply in all types of cell-mediated immune reactions. The tissues in question here may be relatively non-antigenic—they may not have strong tumor-specific transplantation antigens. This may be one reason why they escape destruction.

**N. Levy:** Returning to this question of what a premalignant

cell is: Does the concept the speakers are offering about premalignancy refer to a change in the cell itself? Or is it a change in the overall histologic pattern of the tumor? What role does stromal change play in the change to premalignancy and malignancy?

**Weiss:** If I understand your question correctly, is the so-called premalignant state a quality or property intrinsic to a given tissue, or does it reflect a total interaction between that tissue and the host organism?

**Levy:** Yes, is there a malignant, as opposed to a premalignant, cell and what role does the stromal component play?

**Weiss:** I believe that there is such a thing as a single, intrinsically neoplastic cell, and such a thing as an intrinsically preneoplastic cell, and at the same time I have little doubt but that the "overall pattern"—if by this one means the total contribution of host, environment, and aberrant cells to a given relationship—also has a determinant role in defining the nature of a process leading to neoplasia. It is well known that one can transplant serially, often for many transplant generations, preneoplastic tissues and neoplastic ones with defined characteristics, and find the transplants to remain true to type.

Good examples of such consistency of demeanor by preneoplastic tissues are afforded by the system developed by DeOme and his colleagues at Berkeley for serial transplantation of hyperplastic alveolar outgrowths of mouse mammary glands in the cleared fat pads of isogenic hosts. Over a wide range of different immunologic capacity on part of the host animals, the outgrowths frequently retain their original morphologic and physiologic characteristics. The same thing is often true for already neoplastic mammary parenchyma. In other instances, however, the immune status of the host may, as has been demonstrated elegantly by Dr. Slemmer, impinge significantly on the fate of preneoplastic mammary isografts. Similarly, we have noted that small numbers of fully neoplastic mammary cells may at times lie dormant for a good portion of the life of a host after they are implanted into a young animal. Thus, host and environmental circumstances *can* undoubtedly affect the degree of realization of neoplastic or preneoplastic potential of isogenic and probably as well as of autochthonous clones, and the final picture will depend on the relative strengths of these potentials and of the factors bearing on their realization.

**Slemmer:** Essentially I agree with what Dr. Weiss said. One type of experiment that can help to demonstrate our understanding of neoplasia resulting from transformation in a single cell, which makes that cell malignant, is the experiment I showed, in which the neoplastic premalignant cells depend on normal cells for survival. When these neoplastic cells become malignant, nothing can be done to make these cells dependent again on normal cells. They have gained the capacity for autonomy and they retain that capacity.

On that basis, we say that this is a heritable alteration in that cell population. It doesn't depend on whether normal cells are present or on the relationship of those cells to the stroma. It's an inherent property of the cells themselves.

**S. S. Tevethia:** The question of defining what is pre-



malignant and what is malignant is extremely important, especially in tumor virology, where one wants to know if cells transformed *in vitro* have acquired malignant potential by measuring transplantability.

If one transforms hamster cells *in vitro* with Simian virus 40 (SV40), then morphologic transformation correlates well with transplantability *in vivo*. However, if one transforms BALB/c mouse cells *in vitro* with SV40, the cells show all the criteria of morphologic transformation, loss of contact inhibition, change in karyotype, and loss of growth inhibition. However, some of these cells are not transplantable in the syngeneic mice, even when immunosuppressed. Sometimes one has to grow these cells for up to 50 generations *in vitro* before they can become transplantable. We have to define conditions under which the malignant potential of transformed cells can be demonstrated.

**Black:** I would like to comment on the number of questions raised about precancerous and *in situ* carcinoma and other related situations. I appreciate the problem because we face it every day, and certainly every practicing pathologist and clinician face it.

Observationally, there are atypical alterations which empirically can be recognized and shown to have a higher risk of subsequent association with the development of invasive, clear-cut cancer. In this sense, they are precancerous.

Then there are lesions which we know are cancerous, because they are invasive. In adjacent areas, similar changes may be seen within intact ducts. Those lesions might be called preinvasive foci of cancer.

When you have similar changes without any invasion, you rely on the empirical association between such structural change and the subsequent development of cancer in a greater than average frequency.

If you want to be dogmatic, you say it's *in situ* carcinoma. If you want to be less committal, you say it's precancerous only in a statistical sense.

Let me make just one other point: Given lesions that do truly invade, there seems to be a tendency here to use the term "cancer" as if it were all one package, even in the same tissue. It is not. Cancers in the same location may widely vary intrinsically in their structural appearances, degrees of differentiation, and in their biologic behavior.

With carcinomas of the breast that have highly differentiated nuclei, survival is superior. With carcinomas of the breast that have highly undifferentiated nuclei, some sort of host response is necessary or survival is going to be very poor. These are lesions that will rapidly kill the patient.

Even if we make our definition as rigid as possible and define a lesion as cancer only when it invades and kills the patient, the time it takes for it to do so will differ a great deal depending on variables within both the host and the cancer.

**Slemmer:** I would like to make a comment and ask Dr. Black a question. I am very much impressed by the observation that precancerous lesions may be invaded and inhibited by immune cells, and I am particularly excited by Dr. Black's observation that different lesions, arising in the same woman, may have similar antigens. Dr. Black, do you have any ideas about what type of antigenicity this may be? Is it related to the presence of mammary tumor

virus or possibly the presence of tissue-specific antigens? And could this type of immunity be transferred from one woman to another, or be increased in the woman with precancerous lesions?

**Black:** I wish I could answer the very excellent questions you've asked, but I have no idea, since I don't know, really, whether breast cancer is of viral origin. We have no direct observations on anything of that sort.

**H. T. Wepsic:** Dr. Black, what percentage of breast cancer patients have this infiltration? Was your protocol included in the national cancer program?

**Black:** The percentage in random cases of invasive breast cancer patients showing sinus histiocytosis reactions is slightly under 30%. That's about the same percentage as primary lesions which will show a perivenous lymphoid-cell infiltration reaction.

Diffuse lymphoid infiltration occurs in roughly about 5% of breast cancer cases. However, the incidence approaches 50% in so-called medullary cancer. I also showed that the incidence of favorable types of reactivity will be a function of the stage. The lower the stage, the higher the incidence of such reactivity.

The second question had to do with breast cancer programs. Were you referring to prophylactic chemotherapy?

**Wepsic:** I think there's a program on now by the American Cancer Society in which they are taking several groups of patients and randomizing them to treatment: as to whether or not they have simple mastectomy, radical mastectomy, radical mastectomy with irradiation, and other modes of the treatment. In this study, the pathologist is directly responsible for evaluating the tumor, lymph nodes, etc. Have you collaborated in this project?

**Black:** I think one of the studies you are referring to is that of Dr. B. Fisher and, of course, there is a very large study under the direction of Dr. M. Baum in England. The latter study is using the criteria for assessing reactivity that I have described. I do not know about the other studies. As a pathologist, I do not control the breast cancer patients. They are controlled by the surgeons.

However, I would say this here and now: It has been my advice to my surgeons not to enter, because I do not believe that a heterogeneous disease should be treated by any one method. It's illogical.

**Wepsic:** Could you comment on inflammatory carcinoma of the breast tumor where one observes tumor infiltrating the dermal lymphatics? What do you think might be going on in this disease, in which you probably have little reactions, as you have described?

**Black:** Inflammatory carcinoma of the breast, as you know, is a clinical diagnosis. The pathologist merely says: "Yes, there is diffuse intradermal lymphatic involvement. Therefore it is structurally consistent." But there is no specific pathology for inflammatory carcinoma of the breast.

We have had a chance to perform skin tests on a few of these. These tests gave negative results in terms of reactivity.

**J. Gutterman:** Dr. Weiss, would you elaborate more on a point you made in your summary on the possible role of tolerance in decreasing immune surveillance in the early preneoplastic state?

**Weiss:** What I had in mind is the following: Faced with a massive load of foreign tissue, the immunologic mechanism

of most animals will be specifically incapacitated. Even for some time after removal of a large mass of antigenic cells, the host remains in a state of at least partial tolerance; that is why specifically acquired heightened resistance against antigenic grafts can frequently not be detected for some days or weeks after removal of the initial implant. When, in contrast, the alien tissue is very small, as in the early stages after the occurrence of a preneoplastic or neoplastic transformation, such "overload tolerance" is not, by definition, a factor. This does not, of course, imply that preneoplastic cells may not be shielded in the very early stages of their existence, when they still share with normal cells many characteristics, including antigenic ones, by the same mechanisms of self-tolerance which protect normal tissues from autoimmune attack; this is a very different situation, however, from the tolerance induced by overwhelming quantities of an antigen.

**G. B. Mackaness:** For immunologic surveillance to operate, it is necessary for antigens from precancerous cells to make effective contact with the immunologic apparatus.

Normal tissues contain many antigens which, if removed from the body and reinjected with adjuvant, provoke an immune response. Autoimmune thyroiditis is an example. It is a presumption, therefore, to argue that precancerous cells are exciting an immunologic response simply because we can demonstrate that they contain antigenic determinants which are tumor specific and identical with those of their malignant counterparts. There is a lot of evidence, such as the example I have just quoted of autoimmune disease, to suggest that this is not necessarily so. We have encountered another example in mice infected with highly antigenic microorganisms, such as the *Listeria monocytogenes*. After aerogenic infection of the lung, small numbers of the organism produce a smoldering infection without exciting an immune response until some of them escape from the lungs and enter lymphoid tissue. Not until the organisms can be detected in the spleen is a response initiated that can eradicate the primary infection in the lung. For these reasons, I question whether the mere presence of tumor-specific antigens on tumor cells is enough to enable a surveillance mechanism to operate. I wonder, in fact, whether you are right in thinking that immunologic surveillance operates most efficiently in the initial stages of carcinogenesis.

**Weiss:** I fully agree with you, Dr. Mackaness. The mere fact that a tissue possesses a unique kind of antigenicity, either qualitatively or quantitatively, does not mean one can immediately, therefore, expect an immune attack—but the possibility of such an attack is created!

My own view, in very general terms, is that the moment there occurs any imbalance in the normal equilibrium, spatial or physiologic, between a given tissue or cellular system and either its locally draining lymphatic tissue or lymphatic tissue elsewhere in the body, the chances of an immunologic reaction increase. I view the entire area of autoimmune disease as a manifestation of the breakdown of such equilibria, occurring at random with age, and after acute infectious-disease episodes or contact with other cytopathogenic agents.

The moment one deals with a preneoplastic development, one finds either qualitative or quantitative changes in tissue composition. Even the mere *presence* of 5 or 10 times the

number of cells normally present at a given site may break a physiologic or an anatomic boundary. Thus the fat pad of a mouse filled with even a "mildly" preneoplastic tissue constitutes a very different mass of antigen (even of normal histocompatibility antigens, to say nothing of any organ-specific ones) than if one looks at normal virginal or even lactating mammary gland of purely normal tissue.

To put it another way, the mere fact that a tissue or a cell possesses an antigen not normally present, or has more of it, does not by itself make for an active immune response. But, if the changes are qualitatively or quantitatively fairly deviant from normal—and what fairly deviant is would probably depend on the individual host and on the tissue involved—then I would expect such a change.

**Slemmer:** As regards the immunizing capacity of antigenic lesions *in situ*, the studies of Billingham and Medawar,<sup>1</sup> of Woodruff and Woodruff,<sup>2</sup> and of Merwin and Hill<sup>3</sup> dealing with the transplantation of allogeneic tissues are pertinent.

Billingham and Medawar showed that allogeneic melanocytes may be transplanted from one guinea pig to another. As long as they stay in the epidermis above the basement membrane, the allogeneic cells can grow and spread and apparently fail to immunize the host, though they remain susceptible to immune destruction if the animal is subsequently immunized.

Similarly, Woodruff and Woodruff and Merwin and Hill showed that transplants of allogeneic thyroid gland or Harderian gland into the loose, subcutaneous connective tissues may fail to immunize, while remaining.

All of these experiments, including the studies of the simulated *in situ* lesions I described, have in common the circumstance that the antigenic cells are separated from the body by an intact basement membrane.

I have the feeling that immunization may be delayed until the cells actually invade the basement membrane, and then the lesions may be malignant and resistant to immune destruction.

**Martin:** May I ask Dr. Lappé a question of definition? There may well be a basic surveillance mechanism able to recognize and react to a foreign or altered cell. On the other hand, there is clearly an immune system characterized by an adaptive immune response, production of cytotoxic lymphocytes, antibody, etc. Invertebrates lack what we consider to be an immune system. Yet these cells are clearly at risk to genetic mutations and presumably to neoplasia. Conceivably these animals may have a cellular surveillance mechanism. This mechanism may have continued through

<sup>1</sup> BILLINGHAM RE, MEDAWAR PB: Pigment spread in mammalian skin: Serial propagation and immunity reactions. *Heredity* 4:141-164, 1950.

<sup>2</sup> WOODRUFF MF, WOODRUFF HG: The transplantation of normal tissues: With special reference to auto- and homotransplants of thyroid and spleen in the anterior chamber of the eye, and subcutaneously, in guinea pigs. *Phil Trans Roy Soc Lond [B]* 234:559-582, 1950.

<sup>3</sup> MERWIN R, HILL EL: Fate of vascularized and non-vascularized subcutaneous homografts in mice. *J Natl Cancer Inst* 14:819-839, 1954.



evolution and still be present in higher animals. If this type of mechanism exists, it might be more useful to have it distinguished from the vertebrate immune response.

**Lappé:** Unquestionably in other animals there are systems that appear to modulate—and I am using the term in its embryologic content—the expression of normalcy in tissues. I'm thinking particularly of the work of Drs. F. Seilern-Aspang and K. Kratochwil,<sup>4,5</sup> who clearly showed that, in the newt, tumors can differentiate if they are juxtaposed to an appropriate modulating mesodermal tissue.

The immune apparatus may well represent a system which has taken over this type of surveillance as a secondary function. I don't see the value of excluding other modulating or regulating forces. Certainly hormones work; certainly clearly nonimmune mechanisms affect the growth or regression of tumors.

**A. K. Gershon:** Some studies have shown that lymphocytes will not react as well with DNA synthesis or blast transformation against xenogeneic tissue. However, it's much harder to get a xenogeneic graft to last in an animal than an allogeneic graft. So there is a lack of correlation between the blast transformation and the immune response.

**Weiss:** With regard to the factor of graft survival, two points should be made. First, obviously when one deals with a xenogeneic graft, one deals with a variety of non-immunologic circumstances that pertain to the ability of the foreign tissue to take. Secondly, I did try to make the point that the possibly more restrictive ability of cellular reactivity to a very distant antigen may not be paralleled by such a restriction in terms of circulating antibody formation, and free antibodies may make a major contribution to the rejection of a xenograft, especially of skin.

Thus, when one deals with a xenotypic graft, rejection may very well represent a combination of specific humoral antibodies, some cellular reactivity, and nonimmunologic circumstances. In contrast, allograft rejection is much more prominently an immunologic event, in which the cellular component may be of particular importance; this may be especially true for autochthonous preneoplastic cells. Moreover, if one looks at *in vitro* systems, one would be focusing much more on the purely cellular reactivity!

**Gershon:** In that regard, if you just take tumor cells as a graft, xenogeneic tumors also grow less well than allogeneic tumors, are less well enhanced, and are harder to grow in immunosuppressed animals; also, the growth is inhibited very early, before you would expect antibodies to play a role. Probably the cellular mechanism is involved, because a more complete immunosuppression can get xenogeneic tumors to grow somewhat. It seems more likely that the xenogeneic tissues are rejected by immunocompetent cells—but more rapidly and without the need for DNA synthesis. Tumor cells are also, as you know, very hard to suppress with antibodies.

**Weiss:** I am sure that we still have much to learn about the relative cellular immunologic capacity of animals against spectra of xenografts, allografts, and autochthonous neoplastic and preneoplastic variants!

I should like to make only a few additional points in answer to Dr. Gershon: For one, early inhibition of xenogeneic tumors does not necessarily preclude an important participation by early antibody, of IgM type. Second, there

may well be an additive or even synergistic effect between such antibody and lymphoid effector cells; many workers have suggested this. Third, little, if anything, is known regarding the relative susceptibility of *preneoplastic* cells to immune lymphocytes and specific immunoglobulins; both types of immune elements may play a role, and differentially so vis-à-vis autochthonous, allogeneic, and xenogeneic preneoplastic targets. Lastly, we should consider the possibly nonspecific cytotoxic capacities of transformed lymphocytes. Even if my suggestion has merit only in so far that some allogeneic or autochthonous antigenic cells may cause a more efficient, more extensive, or more rapid blast transformation of host lymphocytes than some xenogeneic ones, then the blast-stage lymphocytes could, as suggested by Dr. Perlmann and others, act cytotoxically against cells in their vicinity—in other words, a cytotoxic event triggered specifically and then magnified nonspecifically.

Perhaps most pertinently, I should emphasize that the suggestion I made for a possibly more effective immunologic attack on target cells not too far removed in antigenic nature from the host is most assuredly very speculative; and it may be more valid for antigens of allogeneic origin differing from each other in their "distance" from the self of the host than for a comparison between allogeneic and xenogeneic ones!

**M. N. Teller:** There is much information in line with what Dr. Gershon has said. I remember many years ago when we were transplanting human tumors into rats. Within 2 or 3 days, in a normal, young, weanling rat, we would see just the necrotic tissue left from what we had transplanted; yet, when we injected cortisone, these tumors would grow to the size of your fist. There is a strong possibility that this is a cell-mediated type of rejection because of the rapidity of rejection. This occurs too with another xenogeneic tumor transplant, such as the mouse Sarcoma 180 and the rat. If you don't treat the rat, what will happen is that, within 48 or 72 hours, nothing will be visible other than the necrotic tissue, except that occasionally one finds a small island or two of growth. But injection of cortisone at the time of tumor implant will result in growth of tumor to a large size.

**Weiss:** Can you rule out that this cortisone effect is not manifested in terms of IgM antibody formation?

**Teller:** I can't rule it out because we have not done any measurements.

**G. W. Santos:** If we give the rat an injection of a standard number of human lymphocytes, then label these lymphocytes with chromium, do a cell-to-cell interaction, look for chromium release, and then repeat the experiment using an AGB-incompatible to immunize, we always get higher release in the xenogeneic system.

Again, I know the MLC data, but I don't quite under-

<sup>4</sup> SEILERN-ASPANG F, KRATOCHWIL K: Induction and differentiation of an epithelial tumour in the newt (*Triturus cristatus*). *J Embryol Exp Morphol* 10:337-356, 1962.

<sup>5</sup> ———: The relationship between regeneration and tumor growth. *In* Regeneration and Related Problems. Amsterdam, North Holland Publishing Co., 1965.



stand it when it's so easy to immunize to xenogeneic cells and in fact get such tremendous reactivity.

You mentioned that in the xenogeneic situation you were told that there wasn't as much chromium released. I presume these were from unsensitized situations.

**Slemmer:** This discussion stems from the hypothesis advanced by Dr. Weiss in his Summary that the immune response of the host may be less effective against malignant

variants because they may have very "deviant" antigenicity as compared with premalignant precursors.

My studies and those of Dr. Lappé have clearly shown that malignant variants from premalignant tissues have antigenicity similar to, or, if different at all, slightly less than the premalignant precursors. On this basis, the host immunity might be expected to be more effective against premalignant rather than malignant tissues.

### **SESSION 3**

#### **Antigens in Neoplastic Tissue**

**Chairmen: Anthony J. Girardi and Robert W. Baldwin**





## Introduction: Antigens in Neoplastic Tissue<sup>1</sup>

**Anthony J. Girardi, Ph.D., The Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania 19104**

Antigenic expression in neoplastic cells results basically from the transcription and translation of nucleic acid sequences which differ from those ordinarily expressed in normal cells. Unique specificities may result from varying the secondary structure or from shifting the profile of proteins already formed, but even these events may have controls at the genetic level. The new nucleic acid specificities may occur from re-expression of de-emphasized genes, from alteration of the host cell nucleic-acid sequences (*e.g.*, frameshift mutations), or by introduction of new species of nucleic acid molecules into the cell. The antigenic shift may reflect an increase or decrease in a given component or an aggregation of the molecules in the final product. Some of the antigens expressed may have known measurable functions (*e.g.*, enzymes), but others may be involved with cell structure or have unknown functions. Similarly, they vary in location: Some are in the nucleus and cytoplasm or associated with membranes, and others circulate outside the cell. They may play an important role in host-tumor relationships, inducing either rejection of the abnormal tissue or immunologic enhancement of its growth.

*Is the potential for all this information present in normal cells?* Similarities shared by fetal tissue and tumor cells have been found in a wide range of tumor-bearing species, including man. Represented are tumors of most histologic types, either produced by a wide variety of carcinogenic stimuli, such as viruses, chemicals, and irradiation, or having an unknown etiology. This has suggested that "retrogenetic expression" is characteristic of all tumors and that new antigens would thus be a product of those gene sequences normally expressed during some phase of embryonic or fetal life and de-emphasized in normal adult tissue. Certain of these carcino-fetal antigens seem to play a role in rejection of some types of tumors; in addition, autosensitization against tumor cell antigens is known to occur as a result of normal pregnancy. The fetus is perhaps an example of a rapidly growing "foreign" growth; understanding the "immunologic dialogue" between mother and fetus would increase our understanding of the immunologic balance between host and tumor and of how immunologic signals perhaps play a role in normal differentiation.

Of interest concerning fetal-tumor tissue relationships are the observed phenomena of specific resistance stimulated by tumors and, in contrast, the broad cross-reacting response of active fetal tissue. Perhaps specific, repressed, genetic sites become activated by different oncogenic stimuli, with the result that many tumors may vary from each other in antigenic re-expression, but would cross-react with fetal tissue, since fetal tissue would express all antigenic sites normally.

*Are the new antigenic specificities induced by foreign nucleic acids introduced into the cell?* Viruses introduce into the host cell nucleic acids which code for a variety of viral structural and nonstructural proteins, enzymes, cell membrane components, etc. These viral agents may also function through activation of host nucleic acids to allow expression of antigens that are normally repressed. Recent findings, obtained with a sensitive technique measuring nucleic-acid reassociation kinetics, have sug-

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<sup>1</sup> Presented at Conference on Immunology of Carcinogenesis, held by the Oak Ridge National Laboratory at Gatlinburg, Tenn., May 8-11, 1972.

gested also the presence of "viral genome sequences" as a normal component of host cell nucleic acid. Furthermore, induction of infectious virus may occur after appropriate treatment, demonstrating a ubiquitous genome. This integrated genome may be expressed to various degrees and at different times during normal embryogenesis and during postnatal life, accompanied by antigenic expression as well.

In avian tumor viruses, an "extra" RNA component is present in those viruses capable of transformation while it is absent in nontransforming viral particles. What is the function of the protein that is specified by this RNA component and can it be identified immunologically? In fact, is this protein being detected by methods employed for routine testing (membrane fluorescence, group-specific staining, etc.) when broadly reacting sera are used?

The segmented nature of the genome of the RNA virus permits segregation of its information (*e.g.*, the loss of the "transforming RNA" mentioned above) and similarly may permit specific complementation with information from other viruses in the same cell, or from the host cell itself. This "transducing" principle may even be responsible for newly acquired oncogenicity and antigenicity in viral progeny previously nontransforming in nature.

*Changes in host nucleic acid sequences.* An antigen present in a chemically induced tumor may be unique for that tumor because the mechanism of carcinogenesis in this instance may involve a frameshift mutation resulting in an almost infinite number of antigenic specificities at the cell surface. This topic will be discussed more fully by Dr. M. Basombrío in this session.

In addition, chemical action could conceivably activate repressed fetal antigens, either by acting at the level of the nucleic acid or because of a metabolic shift following the transforming event. Furthermore, the carcinogenic stimuli may activate integrated viral genomes with yet another class of antigens being induced.

Depending on their location and function, new or activated molecules may be important or unimportant in the scheme of induced tumor immunity. Tumor-immune cross-reactions might be less apt to occur when the critical antigens are those induced by the frameshift mutation rather than when a virus-induced or fetal antigen is strategically placed in the cell as a tumor-resistance antigen.

Cocarcinogenesis by chemicals and viruses could evolve through a mechanism whereby the virus creates an enzymatic excision in the nucleic acid of the host cell. This is possible even with enzymes of nontransforming virus that can "nick" the host genome, resulting in additional sites for introduction of the chemical. Since RNA viruses also specify enzymes for the induction of DNA repair, this could result in rapid fixation of the early steps leading to mutation. Antigenically, this cell may not differ from those cells induced by carcinogenic chemicals alone, unless the viral genome persists in an expressed form.

*Host genetic control.* Genetic control of resistance and susceptibility has been demonstrated for both the avian leukemia-sarcoma complex and the murine leukemia viruses. The host cell controls may be active through specifying the viral host range at the cellular level (as with the NB or *Fv-1* locus). Also they may determine the level of antigenic expression of a viral genome present in a cell, as exemplified by the 2 dominant genes in AKR mice; both genes are required for expression of complete virus, with one gene controlling the expression of the group-specific antigen. The genetic controls may be more complex, with one locus controlling the expression of a second locus, as with the *Fv-1* and the *Fv-2* loci. Additionally, expression of viral and antigen production and susceptibility to leukemia by Gross leukemia virus are controlled by several genes. The major histocompatibility locus, *H-2*, is known to exert a major influence, the *H-2<sup>k</sup>* allele favoring the occurrence of Gross soluble

antigen, and the  $H-2^b$  allele favoring the occurrence of antibody. However, what was once interpreted as being tolerance in the  $H-2^k$  individuals may be explained by excessive antigen production that results in the formation of antigen-antibody complexes responsible for the lack of circulating antibody. Thus the host genetic control may be expressed in several ways, including immunity induced against disease and control of infection, or transformation, at the cellular level. The interplay of host genetics and immune expression will receive the attention of our first speaker, Dr. O. Stutman.

*Exploitation of antigenic differences between normal and malignant tissues by immunotherapy.* This exploitation has matured to a more exacting science, accompanied by an interesting cycle of experimental approaches. Transformed or tumor-derived cells removed from the host can serve as the immunizing inocula after appropriate inactivation to reduce their oncogenicity. The careful extraction and characterization of purified components of tumor cells are making possible quantitative immunobiology, with the hope of eventually realizing immunoprophylactic and immunotherapeutic goals, possibly even with allogeneic and xenogeneic cell sources. We will hear more on these subjects from Dr. I. Hellström and Dr. E. Leonard. But the most exciting point in the experimental cycle is reached with the return to the tumor-bearing host, where the *in situ* stimulation of the host's immune response may become our most powerful therapeutic tool, without the removal of the tumor and without the preparation of an experimental vaccine. But this is one of the subjects of a later session and no further comment is necessary here.

With these few introductory remarks, I open this session.





# Immunologic Studies on Resistance to Oncogenic Agents in Mice<sup>1, 2</sup>

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**SUMMARY**—The effects were studied of prolonged administration of IgG prepared from rabbit antimouse lymphocyte serum (ALG) on the resistance of strain I mice to the oncogenic effects of 3-methylcholanthrene (MCA) and of certain mouse strains to spleen focus formation by Friend leukemia virus. ALG did not affect latency or overall tumor incidence in C3Hf mice that develop 100% tumors after MCA administration. It increased tumor incidence after MCA administration in strain I mice to 20–37%, compared to a tumor incidence in the controls of 12–14%. It increased tumor incidence to a similar moderate degree in F<sub>1</sub>, F<sub>2</sub>, and backcrosses of I and C3Hf mice. The antigenicity of the tumors appearing in the ALG-treated or control mice after MCA administration was comparable. In the Friend system, ALG could overcome the resistance observed in some mouse strains (termed “relatively sensitive”) but not in other strains (termed “absolutely resistant”); these effects correlated with the host genotype at the *Fv-1* and *Fv-2* loci. These different effects, both in the MCA and Friend systems, could not be accounted for by different immunodepression by ALG in the various mouse strains.—*Natl Cancer Inst Monogr* 35: 107–115, 1972.

THE CONCEPT of immune surveillance includes the idea that genetic or acquired defects of the immune system favor tumor development (1). Thus, one experimental approach for testing the immune surveillance theory has been the induction of immune deficiencies and the subsequent study of the effects of such procedures on tumor development (2–4). This approach has been extremely successful in certain experimental models like polyoma oncogenesis (2, 3) and also, unfortunately, in heavily immunosuppressed human patients (5).

The present paper reports some studies on the

nature of the resistance of strain I mice to the oncogenic action of 3-methylcholanthrene (MCA). It also reports efforts to demonstrate that resistance may be immunologically based. The results suggest that immune mechanisms play a limited role in the genetic resistance to MCA in mice. The present data on the MCA system and the additional remarks on the Friend virus system will clearly indicate that the exact mechanism of resistance to oncogenic agents in mice is still open to critical analysis. There is a great variety of immunologic concomitants related to tumor development but the causal relationship is still undefined.

## MATERIALS AND METHODS

All mice were from our colony, and their origin and care have been detailed in (6–8).

MCA, obtained from Eastman Organic, Rochester, New York, was used without further purification.

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<sup>3</sup> With the assistance of June Smith, Antoinette Hanson, and Judy Brees.

tion. At different doses, MCA, dissolved in 0.1 ml corn oil, was injected subcutaneously into the left groin of each mouse.

Friend virus (Swiss) was kindly provided by Dr. Charlotte Friend, Mount Sinai School of Medicine, New York, New York. Virus preparation and titrations have been detailed (8, 9).

Antilymphocyte globulin was prepared from rabbit antimouse lymphocyte serum, as described by Allison and Law (3), by diethylaminoethyl chromatography. The antilymphocyte serum (ALS) was prepared in adult New Zealand rabbits by 2 intravenous injections of  $10^8$  thymus cells from (C3Hf  $\times$  I)F<sub>1</sub> mice being given at intervals of 14 days and being bled 7 days after the second injection (3, 8). The sera from the rabbits were pooled and heated at 56°C for 30 minutes. The IgG fraction was separated by chromatography. Before use, the antilymphocyte globulin (ALG) was absorbed for 1 hour at 37°C with washed, packed erythrocytes. It was portioned into small samples and frozen at -70°C until use. Before injection, it was diluted 1:4 in buffered saline. IgG from normal rabbit serum was used as control for the ALG in all experiments.

Tolerance to rabbit IgG was induced by the intraperitoneal injection into mice, 1 and 10 days old, of 5 mg high-speed centrifugates of rabbit IgG, free of aggregates (3).

ALG treatment consisted of the intraperitoneal injection of 0.25 ml of the 1 : 4 diluted ALG in 4 doses for the 1st week and then of 0.25 ml of this dilution once a week for the lifespan of the mice. In all experiments reported in tables 1-4, these injections were started when the mice were 30 days old. MCA was administered subcutaneously in doses ranging from 0.20-2.00 mg when the mice were 35 days old. Controls for the ALG were given injections of IgG prepared from normal rabbits (NIgG) in a similar schedule and dose. Those for MCA were given injections of 0.1 ml corn oil. The animals were inspected weekly for local tumor development. The experiments were terminated 400 days after MCA administration, *i.e.*, when the mice were 435 days old.

For the Friend virus experiments reported in table 8, the ALG administration was different. The various strains all were given injections at birth with ALG or NIgG according to the following schedule: 0.10 ml of diluted ALG or NIgG, given subcutaneously and then twice weekly for 4 weeks

(with the dose for the last 2 weeks being 0.25 ml). All animals were weaned at 30 days of age and given intravenous injections of 0.2 ml of buffered saline containing 20 focus-forming units of Friend virus (8). Nine days later, they were killed. The foci in their spleens were estimated as described by Axelrad and Steeves (9).

For the assay of the immunosuppressive effect of ALG, the following tests were performed: the graft-versus-host capacity of spleen cells (8, 10) and the response *in vitro* of spleen cells mixed with allogeneic cells (11) and agglutinins in serum to sheep red blood cells (7). These tests were performed on 90- and 300-day-old animals treated with ALG according to the schedule described for the MCA experiments. The techniques for the different tests were described previously (7, 10, 11).

The degree of tumor antigenicity was studied as described by Old *et al.* and Stutman (12, 13). The experiments (table 6) consisted of the challenge of preimmunized syngeneic mice with graded doses of viable tumor cells. Growth inhibition of the transplanted tumor indicated the antigenicity of the tumor.

Cellular immunity to the MCA-induced tumors (table 7) was measured by a modified *in vitro* technique described by Jagarlamoodu *et al.* (14). The only significant modification of the technique was that incubation lasted 52 hours and the cultures were subjected to 10 cycles per minute of rocking and a 24-hour static phase at 37°C in 5% CO<sub>2</sub> in air. The lymphocyte : tumor cell ratios used in these experiments were 1,000 : 1. The results were expressed as percent loss of target (table 7). In the experiments in which blocking sera were tested, the target cells were incubated with 0.25 ml undiluted serum for 1 hour, washed repeatedly, and then exposed to the lymphocytes.

## RESULTS

### Effects of ALG on Oncogenesis by MCA in Mice

Table 1 shows the effects of prolonged administration of ALG on tumor development after MCA injection in C3Hf mice. With both MCA doses (0.20 and 2.00 mg), tumor incidence and tumor latency period did not differ significantly between the ALG-treated and the control animals given injections of NIgG. However, tumor appearance was slightly accelerated when the 2 doses of MCA were



TABLE 1.—Effect of MCA dose or treatment with ALG on local tumor development in C3Hf mice 400 days after MCA administration

MCA dose (mg)	Treatment*	Tumors†	Percent	Latency period (days $\pm$ SE)
0.20	None	50/51	100	133 $\pm$ 7.6
.20	NIgG	99/100	100	130 $\pm$ 6.6
.20	ALG	110/110	100	129 $\pm$ 7.2
.20	NIgG†	33/33	100	114 $\pm$ 5.2
.20	ALG†	29/29	100	110 $\pm$ 5.0
2.00	NIgG	40/40	100	110 $\pm$ 4.3
2.00	ALG	50/50	100	112 $\pm$ 6.1

\* ALG or NIG treatment was started when mice were 30 days old and was continued weekly for the lifespan of the mice (see "Materials and Methods" for description of tolerance induction to rabbit IgG and ALG dosage). MCA in 0.1 ml of corn oil was injected subcutaneously into 35-day-old mice.

† Total number of tumors at 400 days after MCA administration/total number of animals studied. Results were pooled for male and female mice, since tumor incidence and latency periods did not differ significantly between subgroups.

‡ No pretreatment at birth for tolerance induction to rabbit IgG.

compared (130 days with the 0.20 dose and 110 days with the 2.00 dose). With prolonged administration of ALG, without pretreatment for tolerance induction to rabbit IgG (table 1, lines 4 and 5), tumor development was accelerated (114 and 110 days, respectively, for the NIG-treated and ALG-treated mice receiving 0.20 mg MCA). These last results indicated that some of the side effects of prolonged foreign-protein administration may be important and not related to special properties of ALG. Although I did not study systematically the effects of prolonged ALG administration, all animals not treated for tolerance induction had severely damaged kidney glomeruli, which suggested development of an antigen-antibody-complex disease; in addition, 25% of these animals had amyloidosis of liver, spleen, intestinal villi, and ovaries. These kidney damages were observed in mice given injections of ALG and of NIG; they were not observed in normal C3Hf mice of similar ages (15). Presence or absence of these side effects may explain the discrepancies regarding the effect of ALS on carcinogenesis by MCA in mice (16–20).

Table 2 shows the results of ALG administration on tumor incidence after 3 different doses of MCA in I mice, which are relatively resistant to MCA carcinogenesis (6, 12, 27). In all mice, ALG treatment increased tumor incidence (lines 3, 5, and 8) regardless of the MCA dose. However, the effects of ALG were not clearly related to MCA dose; thus the group given injections of 0.20 mg MCA

had a higher increase in tumor incidence after ALG administration than the group given injections of 0.50 mg MCA. In all instances, mice treated with ALG, compared with the NIG controls or with untreated animals, had approximately a doubling of their tumor incidences. Tumor development was slightly accelerated in all mice of the ALG-treated groups. In the groups given injections of 2.00 mg MCA, the incidence of lung metastases was studied. All the animals were killed when their tumors reached 1 cm in diameter. Lung metastasis was found 10–20 days after local tumor appearance in 2 of 13 mice in the NIG-treated group (15%), 2 of 17 mice in the untreated group (12%), and 6 of 23 mice in the ALG-treated group (26%). Thus ALG treatment also increased the incidence of metastasis of the primary sarcomas.

Table 3 shows the tumor incidence in different hybrids and crosses of I and C3Hf mice after MCA administration and the influence of ALG treatment. The tumor incidence in the NIG-treated  $F_1$  and  $F_2$  hybrids and in the backcross to I was essentially similar to that previously reported (7) and suggests the multigenic nature of the inheritance of resistance to MCA (table 3, lines 1, 3, 5, and 7). However, ALG treatment increased total tumor incidence, especially in the  $F_1$  and  $F_2$  hybrids (lines 2 and 4) and in the backcrosses given the high MCA dose (line 8). Results found for latency periods generally paralleled those found for tumor incidences. But latency periods did not change after ALG treatment, with the exception of those found among the  $F_2$  hybrids, which had significantly decreased latency periods (lines 3 and 4).

TABLE 2.—Effect of MCA dose or treatment with ALG on local tumor development in I mice 400 days after MCA administration

MCA dose (mg)	Treatment*	Tumors*	Percent	Latency period (days $\pm$ SE)
0.20	None	56/384	14	186 $\pm$ 3.8
.20	NIgG	15/120	12	183 $\pm$ 8.9
.20	ALG	20/60	33	175 $\pm$ 10.4
.50	NIgG	12/97	13	184 $\pm$ 16.1
.50	ALG	12/60	20	170 $\pm$ 12.2
2.00	None	17/120	14	181 $\pm$ 7.5
2.00	NIgG	13/95	14	193 $\pm$ 14.8
2.00	ALG	23/62	37	173 $\pm$ 7.1

\* See footnotes in table 1 for details of MCA and ALG administration. Results were pooled for male and female mice, since tumor incidence and latency periods did not differ significantly between subgroups.

TABLE 3.—Effect of MCA dose or treatment with ALG on local tumor development in crosses between C3Hf and I mice 400 days after MCA administration

Hybrid cross	MCA dose (mg)	Treatment*	Tumors*	Percent	Latency period (days $\pm$ SE)
(C3Hf $\times$ I)F <sub>1</sub>	0.20	NlgG	78/120	65	141 $\pm$ 10.1
(C3Hf $\times$ I)F <sub>1</sub>	.20	ALG	90/90	100	159 $\pm$ 7.4
(C3Hf $\times$ I)F <sub>2</sub>	.20	NlgG	40/90	45	199 $\pm$ 10.5
(C3Hf $\times$ I)F <sub>2</sub>	.20	ALG	70/90	78	157 $\pm$ 12.1
I $\times$ (C3Hf $\times$ I)F <sub>1</sub>	.20	NlgG	42/90	46	169 $\pm$ 8.2
I $\times$ (C3Hf $\times$ I)F <sub>1</sub>	.20	ALG	51/90	57	180 $\pm$ 7.1
I $\times$ (C3Hf $\times$ I)F <sub>1</sub>	2.00	NlgG	27/60	45	155 $\pm$ 10.3
I $\times$ (C3Hf $\times$ I)F <sub>1</sub>	2.00	ALG	48/60	80	152 $\pm$ 11.2

\* See footnotes in table 1 for details of MCA and ALG administration. Results were pooled for male and female mice, since tumor incidence and latency periods did not differ significantly between subgroups.

### Immunodepression by ALG in Mice

Table 4 shows the immunodepressive effects of ALG in C3Hf, I, and some of their hybrids at 90 or 300 days of age (*i.e.*, 60 and 270 days after ALG treatment was begun). Both cellular (graft-versus-host capacity of spleen cells and mixed leukocyte reactions *in vitro*) and humoral (agglutinins to sheep red blood cells) immunities were clearly depressed by ALG in all groups. The degree of the immunodepressive effect of ALG did not differ between the strains, and no discrepancies between tests were observed. Thus the different effects of ALG on carcinogenesis by MCA in these experiments cannot be explained by differential effects

of ALG on the immune response of the treated animals.

The only detectable difference between the groups studied at 90 or at 300 days of age was in the increase in background activity of the spleens in the 300-day-old group when studied in mixed leukocyte cultures. Also, ALG treatment seemed to increase this background activity in both C3Hf and I mice (table 4, lines 4 and 8). A similar increase of spleen background activity with age was observed in normal or thymectomized C3Hf mice (22).

The presence of hemolytic complement in serum (C3Hf mice are *Hc*<sup>1</sup> whereas I mice are *Hc*<sup>0</sup>) did not seem to influence the immunodepressive effect of ALG in the parent strains or in the hybrids (*Hc*<sup>1</sup>

TABLE 4.—Immunodepression produced by ALG treatment in C3Hf, I, and hybrids between these strains

Strain	Treatment	Age at test (days)	Immune response measured*		
			Graft-versus-host capacity	Mixed leukocyte reaction	Agglutinins to sheep red blood cells
C3Hf	NlgG	90	2.12 $\pm$ 0.7	22,600/3,200	8 $\pm$ 0.3
C3Hf	ALG	90	1.10 $\pm$ .4	4,500/2,700	3 $\pm$ .1
C3Hf	NlgG	300	2.00 $\pm$ .8	35,600/8,700	7 $\pm$ .2
C3Hf	ALG	300	1.00 $\pm$ .3	12,100/11,100	2 $\pm$ .3
I	NlgG	90	2.50 $\pm$ .6	19,000/2,200	9 $\pm$ .3
I	ALG	90	1.12 $\pm$ .4	5,900/2,400	2 $\pm$ .1
I	NlgG	300	1.99 $\pm$ .5	22,300/7,400	8 $\pm$ .3
I	ALG	300	1.00 $\pm$ .8	16,000/12,000	3 $\pm$ .1
(C3Hf $\times$ I)F <sub>1</sub>	NlgG	90	ND†	19,000/2,100	9 $\pm$ .6
(C3Hf $\times$ I)F <sub>1</sub>	ALG	90	ND	3,800/3,500	2 $\pm$ .1
(C3Hf $\times$ I)F <sub>2</sub>	NlgG	90	ND	26,500/4,000	ND
(C3Hf $\times$ I)F <sub>2</sub>	ALG	90	ND	6,000/5,300	ND

\* Animals either were killed at 90 or 300 days of age and their spleens used for assay of graft-versus-host capacity and mixed leukocyte reaction *in vitro* or were immunized with 10<sup>8</sup> sheep red blood cells at 90 or 300 days of age and bled 6 days later for determination of antibodies. Five mice were used in each test per group. The results were expressed as spleen indices for the graft-versus-host reactions (indices  $\geq 1.30$  considered positive), as <sup>3</sup>H-thymidine incorporation in cpm for cultures of experimental spleen cells together with mitomycin-treated C57BL cells versus counts of cultures containing experimental spleen cells plus syngeneic mitomycin-treated cells for the mixed leukocyte reaction (11), and as log<sub>2</sub> base of the titers for the agglutinins.

† ND = not done.

is dominant). This lack of correlation was also observed for a much larger number of mouse strains (8). A correlation between presence of hemolytic complement and immunosuppression by ALS was reported (23).

### Immunodepression by MCA in Mice

Our previous studies revealed that MCA had a potent immunodepressive effect in C3Hf mice, sensitive to its oncogenic action, whereas MCA had no effect on the immune response of I mice (6). Additional studies of this phenomenon indicated that the immunodepressive effect of MCA could be dissociated from its carcinogenic activity, by the use of low doses of MCA in C3Hf mice (7). These low doses, although still carcinogenic, did not detectably affect the immune reactivity of the treated mice (7). These findings, as well as the observations that the immunodepressive effect of MCA is mainly detected when certain humoral antibody responses are measured (7) and that MCA has very mild effects on cell-mediated immunity (7), diminish the possible significance of immunodepression by the carcinogen itself on tumor development.

Table 5 shows one example of the lack of effect of MCA on cell-mediated immunity both in C3Hf and I mice, sensitive and resistant, respectively, to its carcinogenic action (6). The response of spleen cells from MCA-treated or control animals in culture with allogeneic cells was quite comparable, thus showing no effect of MCA on cell-mediated responses. Some effect was detected only with high doses of MCA, in the C3Hf mice.

TABLE 5.—Effect of MCA administration on capacity of spleen cells to respond *in vitro* to allogeneic cells

Strain	MCA dose (mg)	Mean <sup>3</sup> H-thymidine uptake in cpm* (experimental/control)
C3Hf	0	22,600 ± 234/3,100 ± 188
C3Hf	0.20	26,400 ± 289/3,900 ± 176
C3Hf	0.50	20,900 ± 333/3,400 ± 129
C3Hf	2.00	16,000 ± 298/4,300 ± 102
I	0	29,800 ± 339/4,100 ± 167
I	0.20	31,300 ± 420/3,700 ± 188
I	2.00	26,000 ± 339/3,300 ± 150

\* Mean uptake in cpm ± SE, 4 mice per group, and each culture in quadruplicate. The results were expressed as uptake for the experimental group ( $1 \times 10^6$  spleen cells from C3Hf or I mice mixed with  $1 \times 10^6$  mitomycin-treated C57BL spleen cells) versus the controls (C3Hf or I cells cultured alone or with mitomycin-treated syngeneic spleen cells). Mixed cultures were carried for 56 hours, and <sup>3</sup>H-thymidine was added during the last 24 hours of culture.

TABLE 6.—Degree of antigenicity of MCA-induced sarcomas in C3Hf and I mice

Strain	Treatment*	Number of tumors tested	Antigenicity†		
			—	+	++
C3Hf	NIgG	17	4	5	8
C3Hf	ALG	16	3	5	8
I	NIgG	15	3	5	7
I	ALG	19	4	6	9

\* All animals were given injections at 35 days of age of 0.2 mg MCA. All tumors tested appeared between 90 and 160 days after MCA administration and were studied in their first to fifth transplantation passage.

† Degree of antigenicity tested in preimmunized syngeneic mice: (—) none; (+) 60–100% regression after challenge with  $10^5$  tumor cells; and (++) 60–100% regression after challenge with  $\geq 10^5$  tumor cells.

### Antigenicity of MCA-Induced Sarcomas

Table 6 analyzes the antigenic properties of the tumors appearing after 0.20 mg MCA in ALG-treated and control C3Hf and I mice. The results were similar to those from our previous analysis of the antigenicity of the tumors appearing in untreated C3Hf and I mice after MCA administration (13). The proportion of highly antigenic tumors was always similar and usually more than 50% of the tumors tested, regardless of whether they were derived from C3Hf or I mice or from ALG- or NIgG-treated animals.

### Concomitant Immunity in I Mice Developing MCA-Induced Tumors

*In vivo* techniques revealed that the few tumors developing in I mice after MCA administration did so in the presence of an intact immune system (24). These studies also revealed that the tumor-bearing I mice showed signs of specific immunity against their own tumor, indicated by the presence of specific immune cytotoxic lymphocytes in their lymphoid tissues and of specific blocking antibodies in their serum (24). Table 7 shows that the same type of results was obtained with the use of an *in vitro* technique. The serum of the autochthonous host could interfere with the cytotoxic capacity of immune lymphocytes (table 7, lines 4 and 5) and also with immune lymphocytes derived from the autochthonous host (table 7, lines 6 and 7). These results were specific for the tumor tested, and no signs of cross-reactivity between MCA-induced sarcomas were detected (not shown in table). On



the other hand, no such immune lymphocytes nor blocking antibodies could be detected in six ALG-treated I mice that developed local tumors; this is being studied in more detail in my laboratories.

### Effect of ALG on Sensitivity to Friend Virus

Table 8 shows the effects of ALG treatment on resistance to spleen focus formation by Friend virus (Swiss) in several mouse strains. Three different effects could clearly be observed, depending on the host genotype at the major loci that regulate Friend virus sensitivity (25). The first effect was the increased sensitivity of certain strains (AKR and CBA/H) that have the "sensitive" allele at both loci after ALG administration. In a previous publication (8), we termed these strains "relatively sensitive." The second effect was the overcoming of the allele for resistance at *Fv-1* in the BALB/c, I, and A/J strains after ALG treatment. These strains were termed "relatively resistant" in our previous study. The third observation was the lack of effect of ALG treatment on the "absolute resistance" of the strains that have the allele for resistance in both loci (C57BL/Ks, C57BL/6, and C57BL/10J). Whether these effects were purely immunologic or acted on other factors, such as target cells for virus replication or transformation, deserves further study. However, quite clearly, a certain type of resistance could not be overcome by effective immunodepression by ALG in mice.

TABLE 7.—Concomitant cellular immunity and blocking antibodies in I mice developing MCA-induced sarcomas

Lymphocytes*	Serum†	<sup>3</sup> H-Thymidine in target (cpm)	Target loss (%)
No	No	32,500 ± 429	—
Normal	No	31,900 ± 333	—
Normal	Yes	32,300 ± 401	—
Immune	No	10,500 ± 202	68
Immune	Yes	30,100 ± 242	7
Autochthonous	No	17,800 ± 312	45
Autochthonous	Yes	32,600 ± 420	0

\* Lymphocytes were derived from syngeneic I mice: normal, immune to the tumor tested, and from lymphoid tissues of the autochthonous host where the sarcoma developed. Results were expressed as remaining labeling in target and as percent target loss when compared with that in controls. For details, see "Materials and Methods."

† Serum derived from autochthonous host.

TABLE 8.—Effect of ALG on sensitivity to Friend virus in mice

Strain	Genotype*		Foci per spleen ± SE†	
	<i>Fv-1</i>	<i>Fv-2</i>	NIgG	ALG
DBA/2	S	S	20.9 ± 1.0	29.6 ± 2.4
NZB	S	S	22.1 ± 1.3	Confluent
AKR	S	S	1.4 ± 0.2	20.8 ± 1.5
CBA/H	S	S	3.6 ± 0.4	23.2 ± 0.9
BALB/c	R	S	0.0	24.5 ± 1.6
I	R	S	.0	29.1 ± 1.4
A/J	R	S	.0	20.7 ± 1.9
C57BL/Ks	R	R	.0	0.0
C57BL/6	R	R	.0	.0
C57BL/10J	R	R	.0	.0

\* Based on *in vivo* (8, 25) and *in vitro* results (26). S = sensitive; R = resistant.

† Results expressed as mean number of foci per spleen ± SE, 9 days after intravenous injection of 20 focus-forming units of Friend virus (Swiss). Six female mice per group.

### DISCUSSION

The use of heterologous ALS or ALG as an immunodepressive agent has been established (27). In addition, some of the side effects of prolonged heterologous ALS administration have been described (28, 29). In our study, tolerance induction at an early age to rabbit IgG or the administration of ALG at birth eliminated the host immune reactivity against the foreign protein in most animals and allowed prolonged immunodepression without detectable side effects.

Clearly prolonged and effective immunosuppression by ALG in C3Hf mice did not significantly affect the tumor incidence or latency period in these mice after exposure to MCA. However, prolonged ALG administration, without prior tolerance induction to rabbit IgG, shortened latency periods for tumor development (table 1, lines 4 and 5), but this effect was not specific for ALG, since controls given injections of normal rabbit IgG reacted similarly. These results indicate a nonspecific effect of the prolonged antigenic administration on the overall well-being of the hosts, expressed as shortened latency periods for tumor development, antigen-antibody-complex kidney lesions, and generalized amyloidosis. Perhaps such side effects can explain the discrepancies between the reports indicating

that ALS decreases latency and increases the incidence of tumors (16-18) and those reports indicating no significant effects (19, 20). One of these papers (20) clearly revealed that ALS treatment was well tolerated by the animals and could be maintained with low doses of diluted ALS.

The effects of ALG treatment on I mice, relatively resistant to the carcinogenic effects of MCA (6), were a doubling of the tumor incidence and a moderate decrease in latency periods (table 2). These results can be interpreted in different ways. For the orthodox supporters of the immune surveillance theory, they indicate that, in the protracted presence of effective immunosuppression, the relative resistance of the I mice to MCA oncogenesis is overcome and tumor incidence doubles. On the other hand, it is also clear that 60% of the similarly immunosuppressed animals given injections of different doses of MCA remained tumor free during their lifespan, which suggests that other factors of a nonimmunologic type are operative. Tumor incidences were also increased by ALG treatment in the F<sub>1</sub> and F<sub>2</sub> hybrids between C3Hf and I mice and in the F<sub>1</sub> hybrids backcrossed to the I strain (table 3).

In summary, the resistance of I mice to MCA oncogenesis is indeed a complex phenomenon, and manipulation of the immune reactivity of the hosts only moderately affects the overall tumor incidence in mice exposed to different doses of MCA. Other factors such as MCA metabolism are being studied in I mice (30).

The results with the Friend virus system (table 8) also point in the same direction: Effective immunosuppression is only a part of the whole story. Immunosuppression by ALG affected only the resistance to Friend virus expressed in certain mouse strains and was completely ineffective in the absolutely resistant strains. The 2 types of resistance (absolute and relative, regarding the effects of ALG administration) correlate with the host genotype regarding the 2 major loci that regulate the resistance or sensitivity to focus induction by Friend virus (25, 26). In this oncogenic system, clearly additional studies should be made on nonimmunologic factors, such as the effect of ALS or ALG on target cells, virus replication, or other control mechanisms. Previously we reported that, in normal mice of the absolutely resistant types, virus replication was minimal (8). The effects of ALS or ALG on other experimental leukemias are also

complex. While in one investigation ALS increased Rauscher leukemogenesis (31), in another the ALS treatment decreased the Rauscher-induced splenomegaly (32). On the other hand, ALS clearly increased the incidence of lymphoma and leukemia induction by Moloney leukemia virus (3, 33). However, ALS decreased mammary tumor development in mice (34), mimicking the effects of thymectomy (35).

These observations indicate that the experimental models of immune surveillance indeed differ intrinsically. Some of these models can be fully interpreted by the regulation of resistance by an intact immune system, as with polyoma oncogenesis (2, 3) or urethan oncogenesis in mice (36). The models of resistance described in this paper require additional interpretation and experimentation to define the nature of the peculiar resistance to the oncogenic agents.

In the ALG-treated animals (table 6), the percent of antigenic tumors was similar in the sensitive C3Hf and in the "resistant" I mice, and the ALG treatment did not facilitate the appearance of tumors with strong antigenicity, as was reported for MCA-induced tumors in thymectomized animals (37).

Incidentally, neonatal or early thymectomy in I mice does not detectably affect either tumor incidence after injection of 0.20 mg MCA or the antigenicity of the tumors appearing in thymectomized animals (Stutman, unpublished data).

From the present and our previous data (24), the immune status of the host apparently does not influence to any degree the development of antigenic tumors in I mice. Thus, our previous work indicates that antigenic tumors develop in I mice without immunosuppression by MCA itself (6, 24) or, as in the present results, in a severely immunosuppressed host with comparable latency periods (with only a modest increase in overall tumors in this last group).

Tumors developed in the presence of concomitant immunity in I mice, as indicated by *in vivo* experiments (24) and with the use of *in vitro* techniques (table 7). Concomitant cellular immunity (37, 38) and the presence of blocking humoral antibodies in serum (38) seem to occur frequently in experimental tumor systems. I mice are no exception. Still, some of the present results are quite paradoxical in regard to the role of blocking antibodies. In I mice ALG increased tumor incidence, while



at the same time it decreased the production of blocking antibodies. This last observation is presently under study.

This same paradoxical situation applies to the immunodepressive effect of the carcinogens themselves (39, 40). Humoral antibody responses mainly were depressed by MCA, whereas cell-mediated immunity was usually unaffected or only slightly impaired (7) (table 5). If this is so, then MCA should inhibit the growth of antigenic tumors, since it will mainly block the production of antibodies (blocking antibodies) but will not affect the cellular immunity. Some of these disturbing effects have been discussed previously (7). MCA acts preferentially on primary IgM responses, and only high doses of MCA act on IgG responses (7), perhaps leaving unharmed the blocking antibody production. This last aspect is being studied in my laboratory.

In summary, the resistance to oncogenic agents in mice is a valid model for the study of the influence of immune functions on tumor development. While the answers obtained have not been definitive regarding the possible immunologic mechanism of resistance, clearly nonimmunologic events may be important homeostatic mechanisms in the mice resistant to certain oncogenic agents.

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# Studies on the Basis for Diversity and Time of Appearance of Antigens in Chemically Induced Tumors<sup>1, 2</sup>

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**SUMMARY**—Each tumor induced by carcinogenic hydrocarbons has a unique and distinct antigenic specificity. The experiments reported were planned to explain why, when, and how this diversity arises. Since the application of one carcinogen to genetically uniform animals results in tumors with multiple antigenic determinants and each determinant remains as a fixed, heritable property of each tumor progeny, the antigens have been attributed to a mutation-like event. A different explanation recently proposed is that, before the carcinogen is applied, a diversity of potential antigens already pre-exists in the normal cells and the carcinogen just magnifies any one of these variants in a neoplastic clone. To distinguish between these possibilities, we cloned low-tumorigenic BALB/c3T3 cells and applied the carcinogen 3-methylcholanthrene separately in diffusion chambers within the progeny of a single cell. The antigens of the tumors induced were still individually distinct, even if they all had recently originated from a single cell, thus supporting the idea that mutation rather than magnification of pre-existing diversity had occurred. In the latter case, the tumors, first cloned and then induced, should have cross-reacted. That the antigens may be induced after carcinogen application but before tumor formation was also studied. Our approach was to see whether isografts of skin treated with the carcinogen 7,12-dimethylbenz [a] anthracene were already antigenic. No such evidence was obtained. Antigen induction is closely related in time to tumor formation and seems to constitute a mutational event.—*Natl Cancer Inst Monogr* 35: 117–124, 1972.

THE STUDIES which I shall discuss pertain to why antigenic polymorphism arises in chemically induced tumors. It is currently assumed that tumor antigens are a genetically determined and transmitted character. I will adopt this assumption

mainly because antigenic specificities remain after many cell generations as a constant property of tumor progenies (1).

When an oncogenic hydrocarbon is applied to normal tissue, several events occur, of which there is a very limited understanding. But these events result in the arising of tumor progenies, each of which has an apparently unique antigenicity. Among several conceivable mechanisms by which this might occur, some we have envisioned as probable and have tried to test experimentally.

*I. Activation of repressed genes.*—The information for all the protein configurations which determine

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each of the tumor antigens might be coded in the genome of normal cells, but in an inactive state. When neoplastic transformation by a chemical carcinogen occurs, a random assortment of these genes might be expressed.

II. *Clonal amplification*.—According to Burnet's theory (2), the tumor antigens themselves might be present in the normal precursor cells. Each precursor cell would have a different antigenic determinant, arising as a result of random mutations in the normal tissues. These antigens would not be in sufficient quantity to be recognized by the immune mechanism until one of them was reproduced and amplified in a tumor clone (2).

III. *Mutation*.—Chemical carcinogens, being also mutagens (3), might induce somatic mutations at the time of neoplastic transformation. These mutations would produce new genic configurations, and one of a myriad of possible abnormal (mutated) antigenic determinants would be heritably expressed.

Let us now consider the testable aspects of these propositions. Proofs relevant to the genetic origin of tumor antigens are mostly indirect, and only exceptionally (4) has it been possible to apply to them the methods of genetic analysis and to study the loci governing their expression. The models conceived in this discussion are thus necessarily based on pieces of evidence which may be indirect or borrowed from unrelated experimental systems.

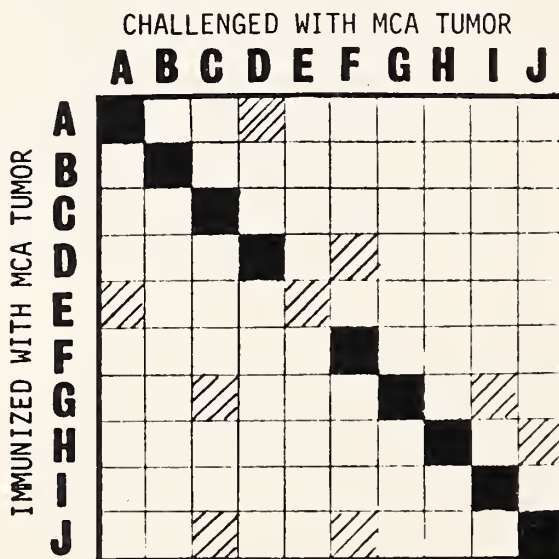
### Possibility of Activation of "Silent" Genes

If the normal genome contains in a repressed state the specific information to produce all eventual tumor antigens (I. *Activation of repressed genes*), then we might expect to find frequently the same repeated determinants in separately induced tumors, indicating that the loci being activated are limited in number. Such seems to be the case, for example, with the carcinoembryonic antigens of the human digestive system (5) or the  $\alpha$ F-globulin found in hepatomas (6) in which the same gene product is detected in many tumors within the system and in embryos. Phenotypic polymorphism can still be generated by a restricted number of genetic loci: The range of varieties can be relatively narrow, as is the case of the major surface antigens of red blood cells (7), or very broad, as is the case of the histocompatibility antigens of mouse and man (8).

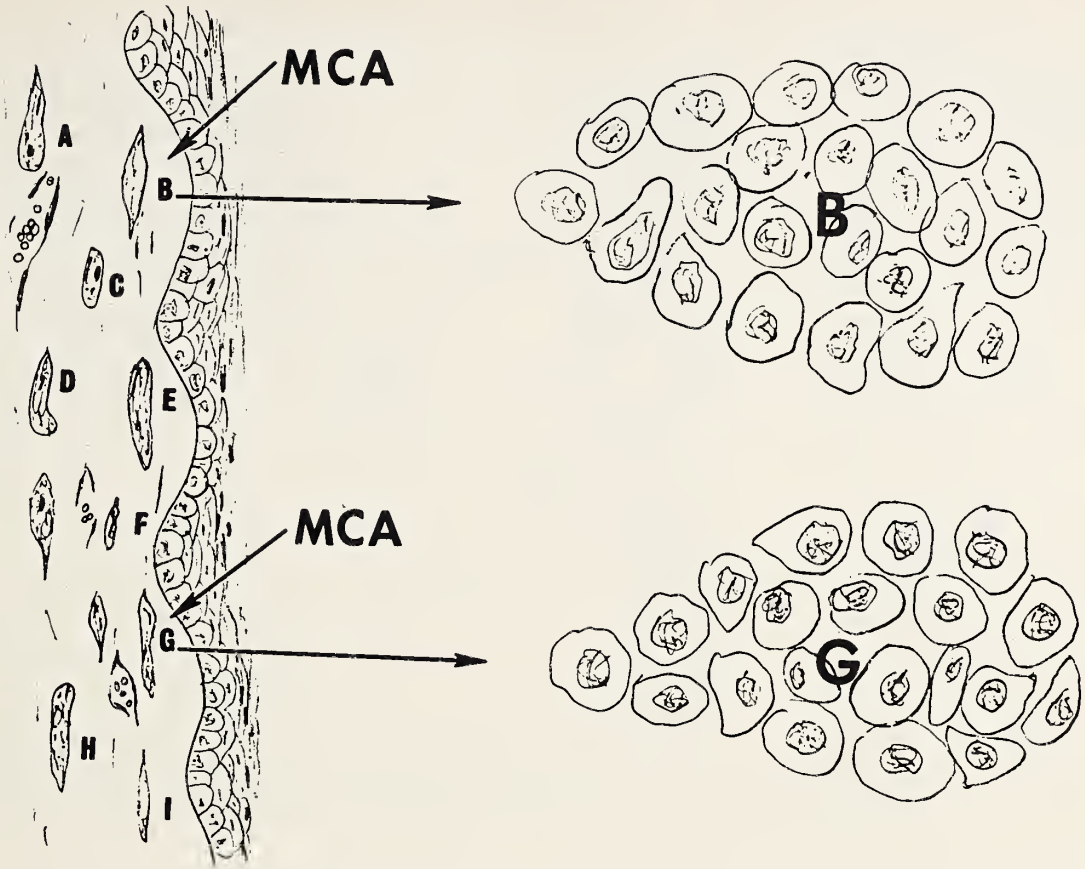
In such systems, cross-matching between randomly chosen individuals may be very infrequent. However, an orderly search attempting to match several samples in all combinations and permutations among them can distinguish between the random occurrence of a myriad of possible antigenicities and a system of restricted antigenic types.

The surface antigens of mouse sarcomas induced by 3-methylcholanthrene (MCA) have also revealed sporadic cross-matching in random tests (9-12). To explore the practical possibilities of typing MCA-induced tumors within antigenic groups, we performed all the 90 possible tests for cross-antigenicity between 10 separately induced tumors (13). Groups of mice immunized with one tumor were challenged intradermally in multiple skin sites with cell suspensions of the same tumor and of 9 other tumors. The cases in which a tumor seemed to immunize against a different one were recorded and retested (text-fig. 1). No reproducible cross-reactions were found; thus the number of possible types was at least 10 and surely much higher. The practical possibility of antigenically typing these tumors was therefore discarded.

By applying similar transplantation methods, we attempted to detect shared antigenicities be-



TEXT-FIGURE 1.—Screening for shared antigenicities among 10 sarcomas induced by MCA. Capital letters: independently induced tumors. Black squares: significant rejection of challenge inoculum. Banded squares: rejection which could not be reproduced. White squares: tested combinations which showed no rejection.



TEXT-FIGURE 2.—Scheme of the clonal amplification hypothesis. Antigenic determinants of tumors (A, B, C, etc.) pre-exist in normal precursor cells. Since each precursor cell contains different antigens, these antigens are insufficient to be recognized by the immune mechanism until clonal amplification occurs in the tumor.

tween a variety of embryonic and fetal mouse tissues (including teratomas) and chemically induced tumors, but we were unsuccessful (14).

The extreme rarity of shared antigenic determinants revealed by these studies suggests that individually specific tumor antigens are the products of somatic mutations rather than of activations of "silent" genes.

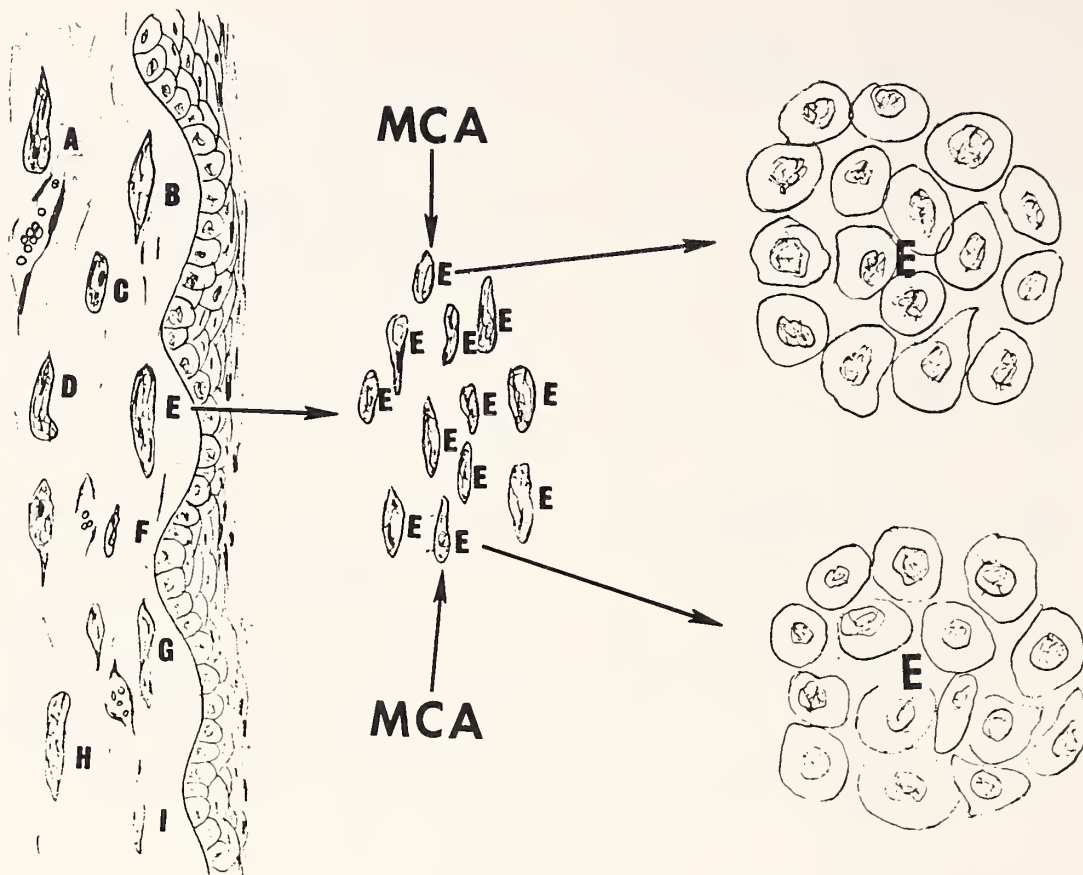
#### The Possibilities of Random Mutation and Clonal Amplification of Pre-existing Antigenic Variants

The hypotheses listed above under II (*Clonal amplification*) and III (*Mutation*) have in common that they both attribute the diversity to mutations. The main difference between them is that, according to II, random mutations occur before the carcinogen is applied and a "latent" mutant is then amplified

(text-fig. 2). According to III, the mutation occurs *after* the carcinogen is applied, perhaps as a direct consequence of it.

These possibilities could be distinguished if one were to isolate a single normal cell, to develop a clone from it, and, within this clone, to induce separately 2 tumors with MCA (text-fig. 3). If II is correct, the 2 tumors induced should cross-react antigenically, since there would be no pre-existing differences to be made evident. If, on the other hand, the antigenic diversity arises by mutations induced by the carcinogen (III), then the 2 tumors should be antigenically distinct, since the carcinogen would induce the diversity.

Our attempts to perform this experiment with primary explants of mouse fibroblasts failed because the cells either became neoplastic spontaneously *in vitro* or stopped growing. However, at least



TEXT-FIGURE 3.—Scheme of experiment to test clonal amplification hypothesis. Cells are cloned before transformation, and 2 tumors are separately induced with MCA within the clone. These tumors should cross-react due to lack of pre-existing diversity.

2 established cell lines from syngeneic mice grow continuously *in vitro* and still produce few tumors when re injected into mice of the syngeneic strain: a line from C3H mouse prostate cells (15) and the BALB/c3T3 line of mouse fibroblasts (16).

By using the 3T3 cell line I could induce tumors separately with MCA within cell clones. Single cells were isolated *in vitro* on pieces of coverslip (text-fig. 4). After small cell colonies had formed on the coverslips, these were transferred into Millipore diffusion chambers, where clones were developed and transformed with MCA.

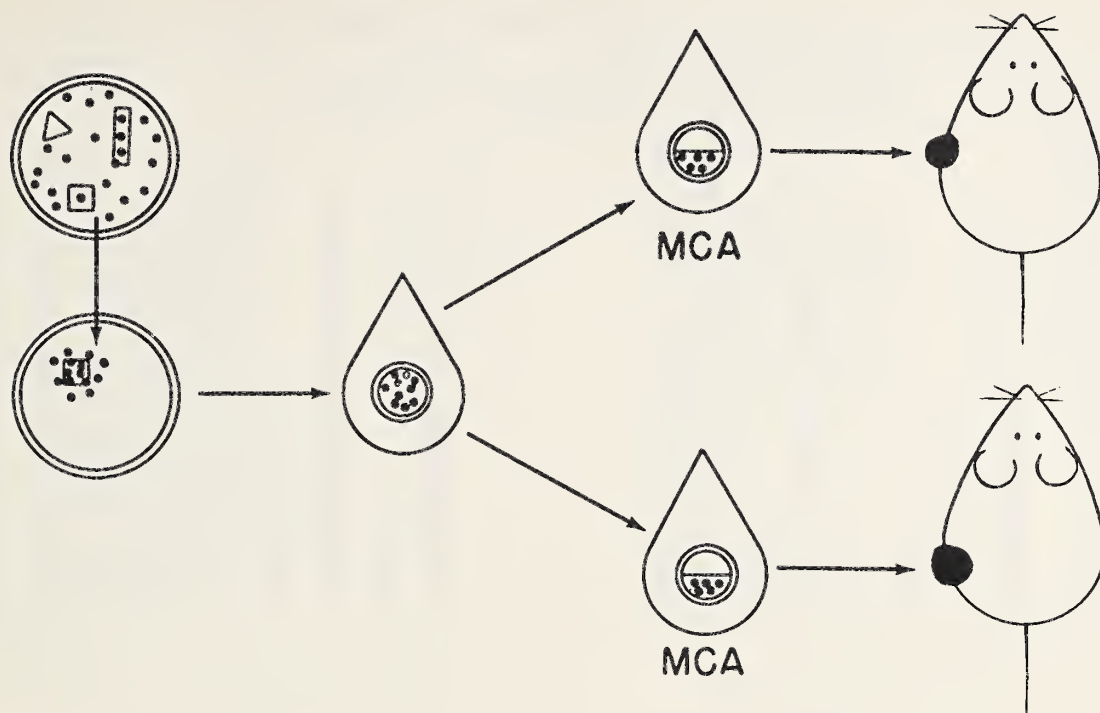
The tumors induced were grown in BALB/cCR mice. The tests for antigenic specificity were performed as indicated above for typing attempts, except the control animals were immunized with a

3T3 tumor variant obtained without MCA or with BALB/c3T3 cells.

I tested 6 tumors from 2 different clones and observed that the immunization with 1 tumor protected the mice against reinoculations of the same tumor but not against reinoculations of other tumors from the same clone (text-fig. 5). Thus different tumors from the same clone still had individual antigenicities.

Similar results were obtained by Embleton and Heidelberger (15) with the C3H mouse prostate cell line, although in these experiments cell transformation was induced *in vitro* and the antigenic specificity of the induced tumors was determined by *in vitro* cytotoxicity and membrane immunofluorescence tests.





TEXT-FIGURE 4.—Procedure used to separately induce 2 tumors within one cell clone. BALB/c3T3 cells were cloned *in vitro*. The clone was developed in a diffusion chamber and then divided into 2 chambers, containing MCA, where tumors were separately induced. These tumors were then grown in BALB/cCR mice and tested for antigenicity.

The results of these experiments are not consistent with the basic mechanism proposed by Burnet (II. *Clonal amplification*): If antigenic diversity results from differences pre-existing in normal cells, then the cloning of cells before transformation should cancel the diversity and the carcinogen would act on a uniform cell population.

However, Burnet's hypothesis would still be tenable if the postulated somatic mutations in normal cells were frequent enough to provide diversity even within the clones of our experiments. But such a high rate of mutation would, on the other hand, pose a theoretical difficulty to the hypothesis: If the diversity is constantly generated in normal clones, why does this process not continue in neoplastic clones? Instead, one variant becomes fixed, and the clone is "committed" to propagate one antigenicity. Burnet suggests that genetic loci can engender diversity in the amino acid sequence of cell membrane proteins. An explanation of why antigenic diversification stops in tumors would then be that the action of such loci is specifically canceled by hydrocarbon carcinogens.

#### Time Relationship Between Neoplastic Transformation and Appearance of New Transplantation Antigens

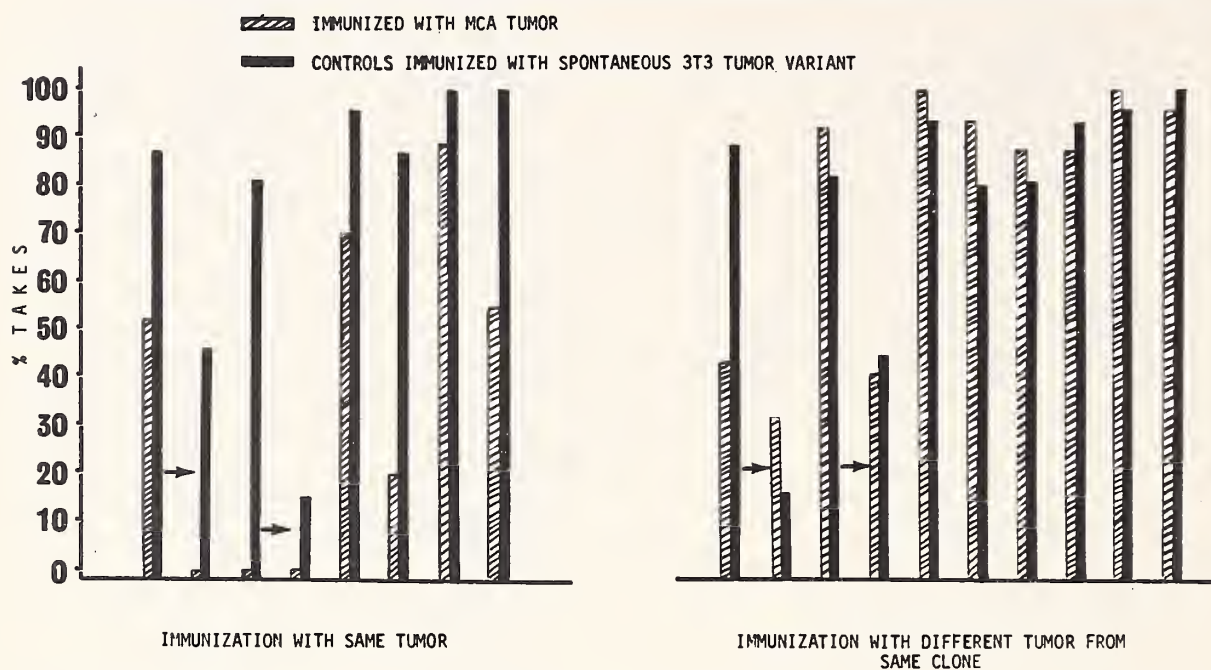
A related problem studied is whether the antigenic change of the cell membrane and the neoplastic change occur independently of each other.

It was first shown in our laboratory (17, 18) that premalignant tumors of the mouse skin and mammary glands already had individually specific transplantation antigens.

More recently, we studied the possibility that antigens may occur before the neoplastic change itself. These studies were prompted by reports (19, 20) indicating that mouse skin treated with the carcinogenic hydrocarbon 7,12-dimethylbenz[*a*]anthracene (DMBA) apparently acquired new antigenicity after 5 days, since the skin was rejected by syngeneic mice.

Attempting to confirm these experiments, we injected intradermally into mice 625  $\mu$ g DMBA, dissolved in 0.05 ml of olive oil. Skin squares taken from the site of injection were transplanted after

## TESTS FOR ANTIGENICITY AND CROSS REACTIVITY BETWEEN TUMORS SEPARATELY INDUCED WITHIN A 3T3 CELL CLONE



TEXT-FIGURE 5.—Each test was performed on groups of 15–20 mice. The ratio between the lengths of the *black* and the *banded* bars indicates the degree of specific immunity. *Arrows* indicate repeated tests. Immunization with same tumor includes tests A–A (immunization with A, challenge with A), A–A, B–B, B–B, C–C, D–D, G–G, and H–H, respectively. Immunization with different tumors includes tests A–B, A–B, B–A, B–A, AB–C, AB–D, C–D, D–C, G–H, and H–G, respectively. *Note* exceptional cross-reaction A–B was not reproduced.

7 days to syngeneic animals. These grafts tended indeed to survive less than control grafts which had been injected only with olive oil. However, even without transplantation, mice had ulceration and scaling of the skin at the site of carcinogen injection. The severity of the lesions was directly proportional

to the dose of carcinogen injected, and the effect resembled that of caustic or toxic substances.

To distinguish between a toxic effect and the presence of neoantigens, we implanted the skin into normal and immunodepressed animals (text-fig. 6). If the failure of the grafts were due to neoanti-

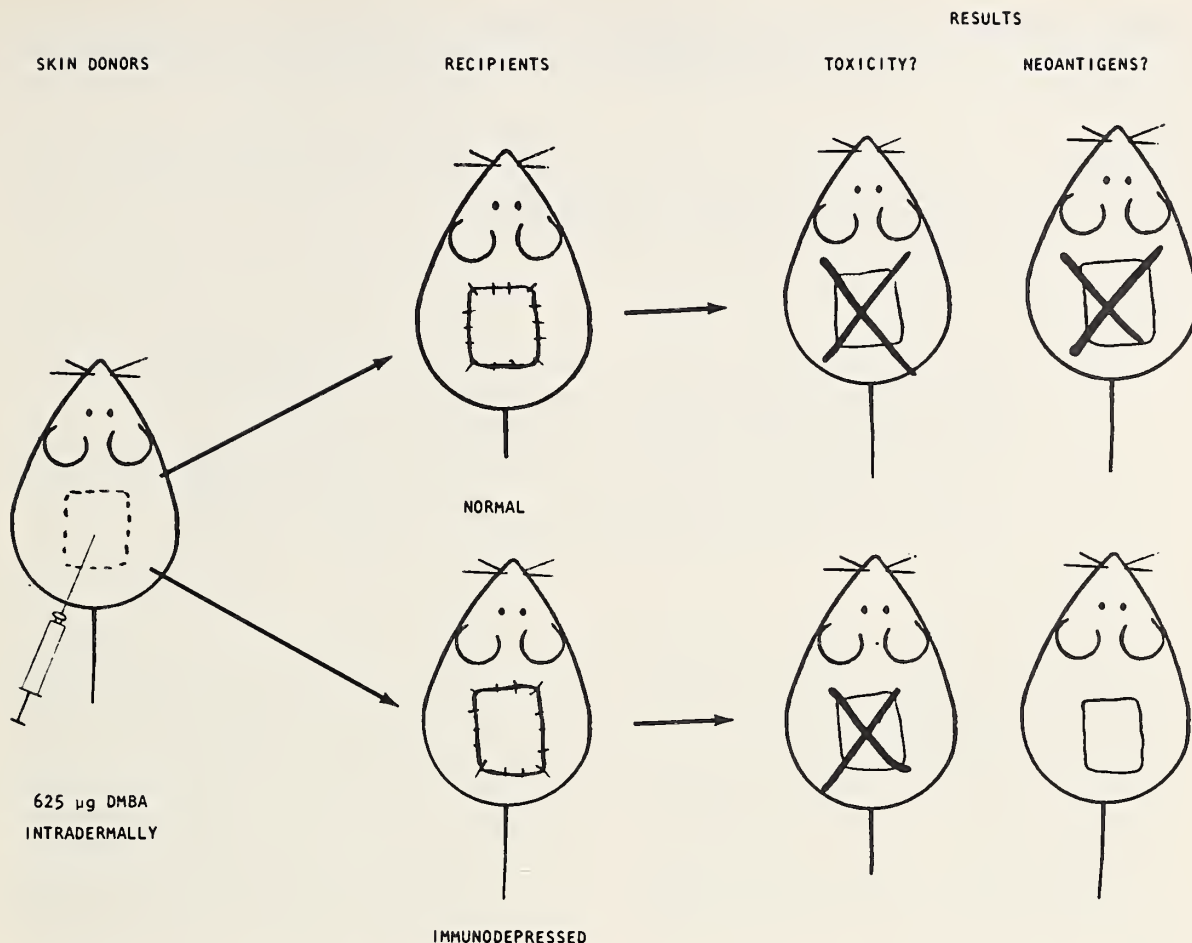
TABLE 1.—Survival of DMBA-treated skin grafts in normal and immunodepressed mice

Experiment No.	Strain	Number of mice	Treatment of graft	Treatment of recipient	Percentage of mice with grafts surviving	
					At 20 days	At 30 days
1	BALB/c	21	Olive oil alone	—	100	100
	BALB/c	21	DMBA*	—	62	62
	BALB/c	21	DMBA	Cyclophosphamide†	57	43
	BALB/c	19	DMBA	Tx—X rad‡	58	52
2	BALB/c	10	DMBA	—	70	60
	BALB/c	7	DMBA	X rad	100	57
3	BALB/c × DBA	14	DMBA	—	86	78
	BALB/c × DBA	15	DMBA	Tx—X rad	80	67

\* DMBA, 625 µg, dissolved in 0.05 ml olive oil was injected intradermally 7 days before grafting.

† Doses of 42.5 mg/kg cyclophosphamide dissolved in saline were injected intraperitoneally every day for 1 week, followed by doses of 21.25 mg/kg every other day for 2 weeks.

‡ Mice were thymectomized and 24 hours later received 450 R of whole-body X radiation.



TEXT-FIGURE 6.—Experiment to distinguish between immunologic and toxic destruction of DMBA-treated skin grafts.

gens, then the grafts should survive better in immunosuppressed recipients. This difference should not be observed if the failure of the grafts were due only to a toxic effect.

The immunodepression of the recipients was achieved by injections of cyclophosphamide or by thymectomy followed by 450 R of X radiation.

In 3 separate experiments, immunosuppression did not increase the survival of DMBA-treated grafts (table 1). Thus our experiments indicate that the apparent rejection of the DMBA-treated grafts is not due to neoantigens, but rather to a direct effect of DMBA.

Observations on the antigenicity of mouse prostate cells treated *in vitro* with MCA (15) also support this conclusion. Of several cell samples taken from MCA-treated cultures, only the tumorigenic ones had new antigenicities.

## CONCLUSIONS

Our studies show that the chances of detecting shared antigenic determinants among chemically induced tumors are extremely low, thus suggesting that the diversity arises from random mutation rather than from activation of fixed genetic information.

To find out whether the antigenic diversity is directly induced by the carcinogen or pre-exists in normal cells, we attempted to detect shared antigenicities in tumors which were independently induced within cloned cell progenies. The sarcomas thus induced were still antigenically distinct from each other, thus suggesting that the diversity does not exist before carcinogen application but arises as a consequence of it.

We could not detect any evidence that the alter-



ations which induce a new antigenicity may not occur simultaneously with those which induce the neoplastic change.

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# Murine Bladder Tumors as Models for Human Tumor Immunity<sup>1</sup>

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**SUMMARY**—Bladder carcinomas of mice and rats are good models for the investigation of immune reactions against tumor-associated antigens of the common tissue-specific type, characteristic of human neoplasms. Bladder papillomas and carcinomas of rats have common antigens, and bladder carcinomas of mice also cross-react, as detectable when the cultivated bladder tumor cells are killed by specifically sensitized lymphocytes.—*Natl Cancer Inst Monogr* 35: 125–127, 1972.

DURING the last 2 years, Dr. L. A. Taranger, Dr. W. H. Chapman, and we have studied immune reactions against urinary bladder papillomas and carcinomas of rats and against bladder carcinomas of mice. The results from these studies are reported elsewhere (1, 2), so we will only discuss here why we think immunologic studies are needed on tumors like murine bladder papillomas and carcinomas, and we will summarize our major findings.

*The need for studies on animal neoplasms such as bladder tumors.*—There is no doubt any longer about whether human tumor-associated antigens exist, since they have been detected in most neoplasms studied, including carcinomas of the colon (3–5), Burkitt's lymphomas (6), neuroblastomas (7), melanomas (4, 5, 8), sarcomas (4, 5, 9), breast carcinomas (4, 5), seminomas (5), lung carcinomas (5), carcinomas of the urinary bladder (10–12), hypernephromas (13, 14), Wilms' tumor (4, 15), and brain tumors (16). Evidence has been obtained for both humoral and cell-mediated immune reactions to the tumor-associated antigens.

A major concern is what the immunologic reactions so far detected against human tumors really mean. This is largely because human tumors, in contrast to most tumors in experimental animals, share antigens common to neoplasms of the same

histologic type—*e.g.*, breast carcinomas share common antigens, as do carcinomas of the urinary bladder—but have different antigens for tumors of various types—*e.g.*, breast carcinomas do not cross-react with carcinomas of the bladder. One may thus wonder whether the antigens detected in human neoplasms are just normal tissue antigens to which the patients become more reactive and to which immunity plays no beneficial role *in vivo*. The fact that a certain amount of cross-reactivity has been detected between some tumor-associated antigens and normal tissue antigens (17) may cause further skepticism as to the function of the common tumor-associated antigens as targets for an immunologic response *in vivo*.

To try to answer this question, one can do different types of experiments. One approach is to search for correlations between immune reactions measured by *in vitro* techniques to find out whether the immune reactions correlate with the clinical behavior of the patients' tumors *in vivo*. Such studies have been done. It has been found that patients with large tumor loads often have less reactive lymphocytes, as detectable by titration of the minimal dose of lymphocytes per target cell needed to produce a significant cytotoxic effect (12, 18). An even more striking difference was observed by tests on the ability of sera from cancer patients to interfere with the cell-mediated reactions: Sera from patients with tumor blocked the reactions, whereas sera from tumor-free patients generally did not

<sup>1</sup> Presented at Conference on Immunology of Carcinogenesis, held by the Oak Ridge National Laboratory at Gatlinburg, Tenn., May 8–11, 1972.

(19), and changes in the blocking serum titers correlated with the clinical response when individual tumor patients were followed (18). A good argument is thus provided that what is measured *in vitro* is, indeed, a meaningful correlation of that immune response believed to play an important part in a patient's fight against his tumor.

Another approach, which may be at least equally important, is to search for animal models of the human situation, in which histologically similar tumors have common antigens. If such common antigens can be detected in some neoplasms of animals by the techniques used for the detection of tumor antigens in man, one could study the *in vivo* roles of lymphocyte cytotoxicity and blocking serum activity against the common tissue-specific tumor antigens.

*The bladder tumor model.*—There is strong evidence for cell-mediated immune reactions against common tissue type-specific antigens in urinary bladder carcinomas of man (10–12). Bladder papillomas and carcinomas of rats and bladder carcinomas of mice are similar to the human neoplasms in their histology and their general clinical behavior. They can be easily induced (20, 21), both by placement of a chemical carcinogen such as 3-methylcholanthrene (MCA) into the bladder and by installment of a foreign body such as paraffin, cholesterol, or silastic. Even very early papillomas, 1 or 2 mm in diameter, can be detected with a transillumination technique (22).

Using as targets cultivated bladder papilloma cells of rats and cultivated bladder carcinoma cells of rats and mice, we studied the extent to which lymphocytes from tumor-bearing or tumor-sensitized animals could destroy cultivated bladder tumor cells (1, 2). Lymph node cells were used as effectors in the experiments on mice, and peripheral blood lymphocytes in the experiments on rats. A microcytotoxicity assay was employed (23), which has been previously used extensively for similar work with other tumors, including those in man (5).

It was found that lymphocytes could destroy cultivated bladder tumor cells derived not only from the tumor against which the lymphocyte donors were sensitized but also from other, independently arising bladder tumors. The lymphocyte effect was specific against bladder tumors. It could be blocked by serum from animals with growing bladder papillomas or carcinomas. Evidence was thus

obtained that bladder tumors of mice and rats behave like those in man, in that they have common type-specific antigens against which cell-mediated immunity can be detected. In agreement with similar observations on skin papillomas and carcinomas of rabbits induced by the Shope virus (24), cross-reactions were found between papillomas and carcinomas.

After rat bladder tumors were found to have a common antigen, it became urgent to study whether the primary induction of bladder papillomas could be delayed (or prevented) by immunization of the rats against tissue containing the common antigen before application of a carcinogenic stimulus. Preliminary results from such studies in rats showed that MCA-induced primary bladder papillomas appeared later after immunization with bladder papilloma tissue, before application of the carcinogen (1).

*Future goals.*—Many questions posed by the recent data need to be answered: Will the common antigens of bladder tumor tissue behave as transplantation antigens in experiments similar to those showing transplantation antigens, e.g., in MCA-induced murine sarcomas? Can the preliminary studies on prevention (delay) of the appearance of rat bladder papillomas be confirmed on a larger animal material and is such prevention due to an induced cell-mediated immunity against a common bladder tumor antigen? Will immunization of animals against bladder tumors damage normal bladder tissue, which would indicate that autoimmune reactions against normal cells have also been induced? Why do the bladder tumors cross-react when several other chemically induced tumors in experimental animals do not? Do they have individually unique antigens, in addition to their common ones, like human malignant melanomas which have both individually unique (25) and common antigens (8), and, if so, what is the *in vivo* role of immunity to the two types of antigen?

Furthermore, since the bladder tumor model involves primary chemically induced tumors (of which there are likely to be human counterparts), it may be relevant in attempts to work out immunotherapeutic schedules. For example, the possible function of “unblocking” antibodies (26) in arresting tumor growth and inducing tumor regression can be investigated in a system which is more similar to the human one than are the systems of the Moloney virus-induced mouse sarcomas (27) or



polyoma virus-induced rat tumors (28), the two neoplasms in which an immunotherapeutic effect with unblocking serum has so far been achieved.

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# Properties of Tumor-Specific Antigen Solubilized by Hypertonic Potassium Chloride<sup>1</sup>

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**SUMMARY**—To extract tumor-specific antigen (TSA) from 2 antigenically distinct lines (designated 1 and 10) of diethylnitrosamine-induced hepatomas in strain-2 guinea pigs 3 M KCl was used. After the TSA was released from the cell structure, it was water soluble, could be precipitated by 2 M ammonium sulfate, and was eluted from G-200 Sephadex in a region from 50–75% of bed volume. The TSA yield was about 10% of the antigenic activity of intact tumor cells. KCl extraction appeared to cause loss or alteration of a large fraction of total antigenic activity, since it was detectable neither in the extract nor in the remaining cell pellet. The residual activity was stable and was used in biological studies. Line-1 and line-10 soluble TSA elicited positive skin tests only in animals immunized with the corresponding tumor cell line. Specificity was also shown *in vitro*: Adding TSA to peritoneal exudate cells from immune donors caused lymphocyte transformation and elaboration of macrophage migration-inhibition factor. Immunizing guinea pigs with line-1 soluble TSA protected against challenge with line-1 tumor cells. Immunizing rabbits with whole tumor cells resulted in production of antibody which could be made specific for the tumor antigen of one line by absorption with cells of another line. Line-1 and line-10 cells each had a line-specific antigen and also had a shared tumor antigen, probably embryonic. Line-10 cells were treated with rabbit anti-line-10 antiserum and then with fluorescein-labeled antirabbit serum. There was an active cellular response to this treatment. Fluorescein-labeled molecules or perhaps the whole antigen-antibody complex was moved within the plane of the cell membrane, collected into large aggregates, and was apparently extruded.—*Natl Cancer Inst Monogr* 35: 129–134, 1972.

FOR THE PAST several years our laboratory has been studying immunologic aspects of diethylnitrosamine-induced hepatomas in inbred strain-2 guinea pigs (1, 2). The ascites forms of these tumors are readily transplantable within the guinea pig strain. I will be discussing 2 antigenically distinct lines, which we designate line-1 and line-10 tumors.

<sup>1</sup> Presented at Conference on Immunology of Carcinogenesis, held by the Oak Ridge National Laboratory at Gatlinburg, Tenn., May 8–11, 1972.

<sup>2</sup> National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

Guinea pigs immunized with either of these lines had specific cellular immunity, as shown by a delayed skin reaction measured 24 hours after intradermal inoculation of the tumor cells. I will describe solubilization of line-1 and line-10 tumor-specific antigen (TSA) and experiments on the biological activity of these TSA's. The last half of the talk will be about specific rabbit antisera to the TSA's. The antisera were raised for use in a quantitative assay of solubilized TSA's; they are also valuable reagents for analysis of antigenic relationships between tumor lines, mechanisms of



TABLE 1.—Delayed skin reactions to line-1 tumor and soluble antigen

Preparation	Skin test result, mm induration		
	Line-1-immune	Line-1-immune	Control
$3 \times 10^6$ cells	$9 \times 9$	$7 \times 7$	$3 \times 3$
$1 \times 10^6$ cells	$7 \times 7$	$9 \times 8$	0
$0.33 \times 10^6$ cells	$3 \times 4$	$4 \times 4$	0
KCl extract from:			
$3 \times 10^7$ cells	$9 \times 9$	$8 \times 8$	$3 \times 4$
$1 \times 10^7$ cells	$7 \times 7$	$6 \times 6$	0

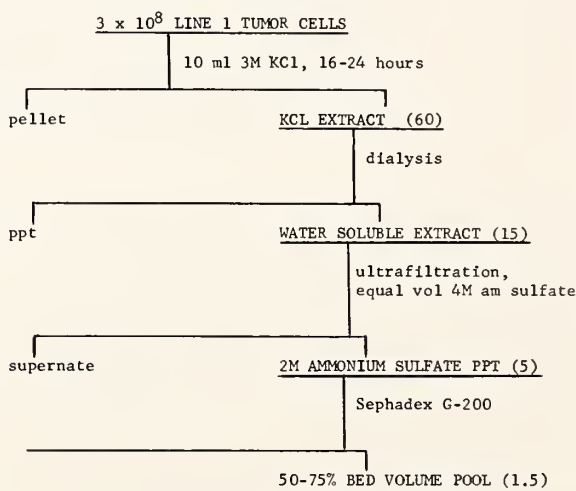
cytotoxic reactions, and molecular movement on tumor cell surfaces.

Our interest in using 3 M KCl for solubilization of tumor antigen followed the report by Reisfeld and co-workers on the extraction of HL-A antigens in hypertonic potassium chloride (3). The use of hypertonic salt solutions to release proteins from native cell structure dates back almost 100 years when the contractile protein myosin was extracted from skeletal muscle (4). In the application to tumor antigens, a key requirement, of course, is a reliable bioassay. We assayed for antigenic activity of extracts by injecting 0.1-ml volumes intradermally into guinea pigs immune to the tumor line and comparing the 24-hour skin test response to that caused by intact tumor cells. Table 1 shows a typical result of an assay in 2 line-1-immune guinea pigs and 1 normal animal. Two perpendicular diameters of the induration at 24 hours were recorded. The top line shows the delayed skin reaction caused by 3 million line-1 cells; the next 2 lines are for serial threefold dilutions. A decreasing response to the lower cell dosage occurred, with one exception in the second guinea pig. Induration in the control guinea pig was negligible. The results with a KCl extract from 3 and  $1 \times 10^7$  cells are shown in the lower part of table 1 and the response to extract from  $3 \times 10^7$  cells was about equivalent to that from  $3 \times 10^6$  intact cells. Thus the TSA yield in this example was about 10%. Table 1 also shows the limits of precision of the skin test method. There was no problem in seeing a progressively decreasing skin test response with falling threefold dilutions of cells; but this was about the limit, and it was not possible, for example, to get consistent skin test differences with serial twofold dilutions of cells. The skin test was sufficiently accurate for testing extracts in the isola-

tion of TSA. Its precision was limited, however, and data on TSA yield were only approximate. Within these limits, TSA yield was about 10%, and the question was what happened to the rest of the TSA.

To get some insight into this problem, we looked at the capacity of cells, treated in various ways, to elicit a positive skin test in immune animals. Cells were blended in a glass homogenizer, frozen and thawed, lyophilized, or extracted with 3 M KCl in the cold for only 15 minutes. The suspension of homogenized cells retained full antigenic activity. The other 3 preparations had only 30–50% of the activity of intact cells. Thus antigenic activity appears to be labile to freezing and thawing, lyophilization, and exposure to hypertonic KCl. The residual TSA activity surviving any of these treatments was stable, however, and it is this material which we have further purified and used in biological studies.

Text-figure 1 is a schematic protocol for extraction of TSA from line-1 cells (5). Ten ml of cold 3 M KCl were added to a pellet of  $3 \times 10^8$  cells. After equilibration on a rocker in the cold overnight, the cell debris was removed by centrifugation. The TSA was in the KCl extract. When the KCl was dialyzed a precipitate formed. TSA activity was in the supernatant. The antigen could be precipitated with 2 M ammonium sulfate and further purified by gel filtration on Sephadex G-200. The total optical density units at 280 nm in the TSA-containing fraction at each step are shown in the parenthe-



TEXT-FIGURE 1.—Flow diagram for extraction and partial purification of line-1 soluble TSA.

TABLE 2.—Specificity of soluble TSA

	Skin reaction (mean radius <sup>2</sup> $\pm$ SD)	
	Line-1-immune	Line-10-immune
$3 \times 10^6$ line-1 cells	$33 \pm 10$	$3 \pm 2$
$1 \times 10^6$ line-10 cells	$2 \pm 2$	$35 \pm 12$
Soluble TSA, line-1	$31 \pm 10$	$4 \pm 3$
Soluble TSA, line-10	$2 \pm 1$	$32 \pm 3$

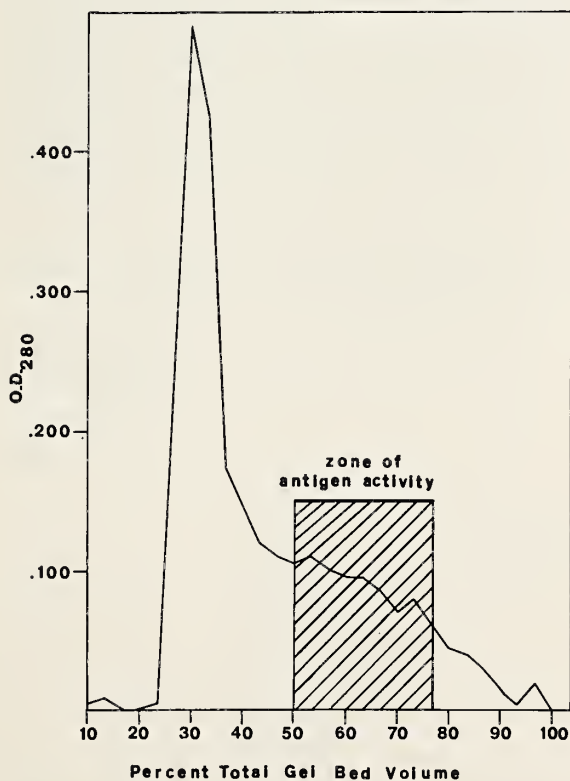
ses, and this indicates the degree of purification obtained, since there was no loss in TSA activity in the steps following the initial extraction.

Text-figure 2 shows the zone of TSA activity in eluates from a Sephadex G-200 column run. The location indicated a molecular weight between 70,000 and 100,000.

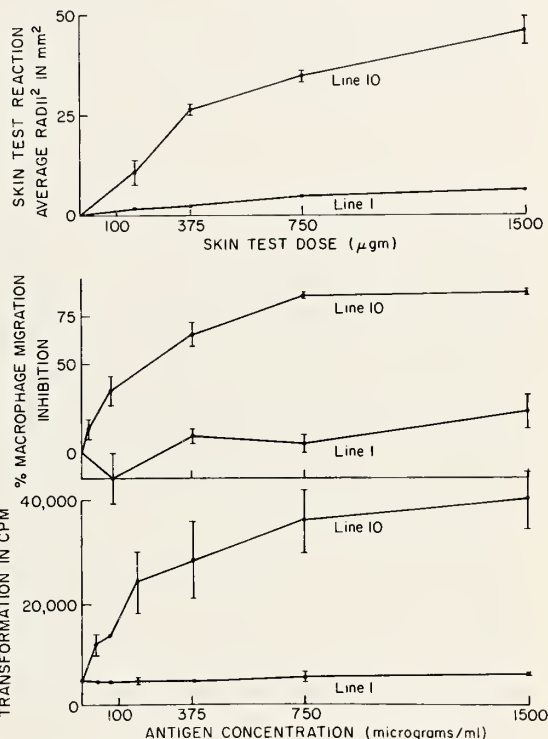
Table 2 shows the skin-reaction specificity of line-1 and line-10 extracts. Skin tests were done in line-1-immune and line-10-immune guinea pigs. Line-1 cells gave positive skin tests with line-1-immune animals and vice versa for line-10. The

bottom 2 lines show the same pattern for soluble TSA from line-1 and line-10 tumor.

The soluble TSA extracts were also tested for their capacity to react in 2 other assays of cellular immunity (6). The results are shown in text-figure 3. In the top panel can be seen a dose-dependent skin test response to line-10 soluble TSA in line-10-immune guinea pigs. There was no significant reaction to line-1 TSA antigen. The second panel shows that line-10 TSA inhibited the migration of oil-induced peritoneal macrophages harvested from

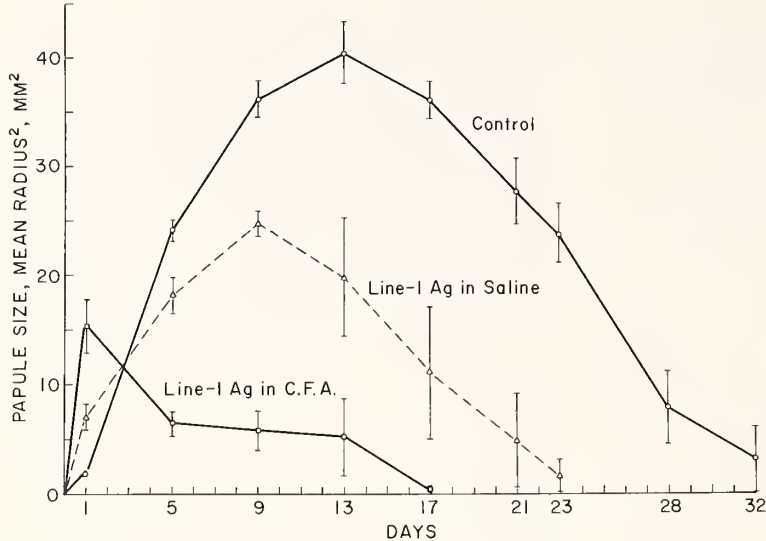


TEXT-FIGURE 2.—Elution of line-1 TSA on Sephadex G-200.

TEXT-FIGURE 3.—Cellular immune response to TSA: skin tests and *in vitro* reactions.

a line-10-immune guinea pig. The bottom panel shows that line-10 TSA stimulated lymphocyte transformation in a cell preparation from a line-10-immune animal. Reactions were positive with as little as 100  $\mu\text{g}/\text{ml}$  in the *in vitro* tests; responses were maximal at about 1500  $\mu\text{g}/\text{ml}$ .

The most critical test of the TSA extract was its capacity to protect guinea pigs against tumor challenge. Guinea pigs were immunized with line-1 soluble TSA in complete Freund's adjuvant, boosted intradermally 3 times at weekly intervals with soluble TSA alone, and challenged 1 month



TEXT-FIGURE 4.—Response of guinea pigs immunized with soluble line-1 TSA to challenge with viable line-1 cells.

after the initial inoculation with line-1 tumor cells intradermally. In control guinea pigs, the injected tumor cells multiplied and formed a measurable papule which subsequently regressed completely (text-fig. 4). The response of guinea pigs immunized with soluble line-1 TSA was a 24-hour delayed-hypersensitivity reaction to the tumor cells, but there was no progressive growth of tumor. Text-figure 4 also shows that immunization with TSA in saline rather than in Freund's adjuvant was not as effective.

The remainder of this talk will be about rabbit antibody to line-1 and line-10 TSA. We were interested in obtaining specific antibody for use in quantitative assay of soluble TSA. After hearing a presentation by Dr. Gail Miller on xenogeneic antitumor antibody (7), we were stimulated to attempt production of specific antihepatoma antibody. Rabbits were given subcutaneous injections of line-1 or line-10 whole cells. Antisera, collected 19–26 days later, were studied. To determine whether the rabbits produced any tumor-specific antibody, we made multiple absorptions of, for example, anti-line-10 antiserum with line-1 tumor cells. Antibody activity of the line-1-absorbed serum against line-1 cells should have fallen to zero; if the serum contained any specific anti-line-10 antibody, activity against line-10 cells should have fallen in the initial absorptions and then leveled off at a steady value. Samples of antiserum obtained during multiple absorptions were equilibrated with tumor cells; the cells were then washed, and their capacity to fix Cl (the first component of

complement), as measured by the Cl fixation and transfer test (ClFT), reflected the amount of complement-fixing antibody in the antiserum. The results are shown in table 3. Looking first at the activity of anti-line-10 antiserum against line-1 cells, we see that the activity of the unabsorbed serum, with a value of  $6 \times 10^9$  Cl-fixing molecules in the  $20 \mu\text{l}$  sample tested, dropped to a negligible value as the serum was absorbed with line-1 cells, normal cells, and guinea pig leukemia cells. The activity against line-10 cells leveled off at a value of  $7.4 \times 10^9$  Cl-fixing molecules. This constitutes quantitative evidence for tumor-specific antibody. The

TABLE 3.—Specificity of anti-line-10 antiserum for line-10 cells after absorption of the antiserum

Antiserum	Absorbed with:	Number of Cl-fixing sites ( $\times 10^9$ ) generated on:	
		Line-1 cells	Line-10 cells
Before immunization Anti-line-10	Unabsorbed	2.2	1.8
	Unabsorbed	6.0	14.0
	Line-1: 4X	1.5	13.0
	Line-1: 12X	0.2	7.8
	Line-1: 13X		
	Liver: 3X	0.1	7.0
	Line-1: 13X		
	Liver: 3X		
	Line-14: 4X	0.04	7.4
	Line-1: 13X		
	Liver: 3X		
	Line-14: 3X		
	Leukemia: 1X	0.02	7.4



TABLE 4.—Absorption of antitumor antibody by soluble TSA

Antibody	Absorbed with:	Number of Cl-fixing sites ( $\times 10^{-8}$ ) generated on:	
		Line-10 cells	Line-1 cells
Antiline-10	Unabsorbed	50	—
"	Line-10 TSA	6	—
"	Line-1 TSA	56	—
Antiline-1	Unabsorbed	—	10
"	Line-1 TSA	—	3
"	Line-10 TSA	—	26

ratio of specific to nonspecific activity (7.4/0.02) was  $>300:1$ .

When we used cell suspensions of normal guinea pig tissues rather than tumor cells for absorption of antiserum, a new finding emerged. Anti-line-1 antiserum, even after 10 absorptions with normal tissue, still showed significant residual antibody activity against line-10 cells. The same thing occurred with anti-line-10 antiserum; residual antibody activity remained against line-1 cells. Since absorption with normal tissue left residual antibody reactive with the other tumor line, we concluded that, in addition to the TSA, line-1 and line-10 tumor cells share an antigen not found in normal tissue. Most residual activity after absorption with normal tissue was abolished by absorption with guinea pig embryo. Thus the shared tumor antigen was probably embryonic.

Now that we had specific antisera, we could devise quantitative tests for soluble TSA. A simple approach was to absorb a fixed quantity of antibody with the TSA to be measured and then determine residual free antibody either by the ClFT test or by a simpler but less precise method. Since complement reagents were available in our laboratory, we preferred the ClFT test because of its precision. An example is shown in table 4. A 20- $\mu$ l sample of specific anti-line-10 antibody, when added to line-10 tumor cells, generated  $50 \times 10^8$  Cl-fixing sites. If the antibody was equilibrated with line-10 soluble TSA first, Cl-fixing activity was reduced to 6. No loss of antibody occurred on equilibration with line-1 TSA. The bottom half of the table shows similar results for anti-line-1 antibody. Residual antibody could also be determined within a factor of 2 by the estimation of fluorescence intensity caused by antibody adsorbed to tumor cells, with the use of a fluorescein-labeled antirabbit globulin reagent.

Finally, I would like to show the response of live line-10 tumor cells to double labeling with antibody. Freshly harvested line-10 ascites cells were equilibrated for 1 hour at  $0^\circ\text{C}$  with specific anti-line-10 antibody. They were then washed and equilibrated for another hour at  $0^\circ\text{C}$  with fluorescein-labeled antirabbit serum. The cells were again washed and examined for fluorescence in dark-field ultraviolet light. They were diffusely bright and outlined by a ring of fluorescence (fig. 1). The picture is consistent with a uniform distribution of fluorescent reagent over the cell surface. Samples of washed cells with both antibodies on them were then placed in tissue culture medium in a  $\text{CO}_2$  incubator at  $37^\circ\text{C}$  and observed at intervals over the next 8 hours. We observed a striking change in the distribution of fluorescence after 1 hour at  $37^\circ\text{C}$  (fig. 2). In many cells the ring outline was gone, diminished in intensity, or broken up. Within the outline of the cell, fluorescence appeared as stippling or as an intensely fluorescent aggregate. The stippling was often in focus in 2 distinct planes, which indicates that it was distributed on the cell surface rather than inside the cell. The fluorescent aggregates sometimes protruded from the cell; this was seen in suspensions of cells as they moved across the field of view. One of these cells is shown in figure 3. Thus the cells respond to the application of the 2 antibodies: the fluorescent molecules, or perhaps the whole antigen-antibody complex were moved within the plane of the cell membrane, coalesced into large aggregates, and appeared to be finally extruded. Anti-line-10 antibody alone, without subsequent application of the second antibody, did not trigger the cellular reaction. We hope that further experiments will determine whether these very recent observations are only laboratory curiosities or are pertinent to tumor cell biology.

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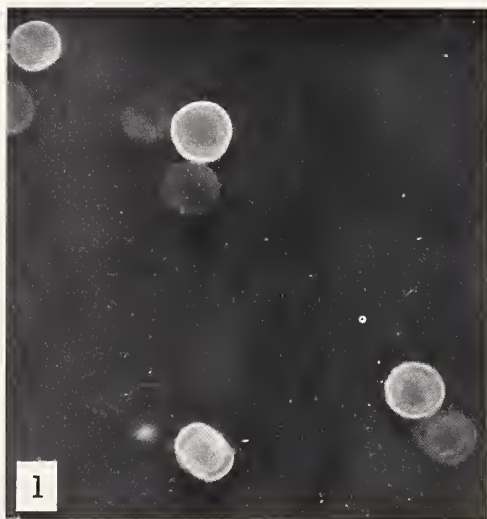


FIGURE 1.—Line-10 cells after equilibration at 0°C with rabbit anti-line-10 antibody and then with fluorescein-labeled antirabbit serum.

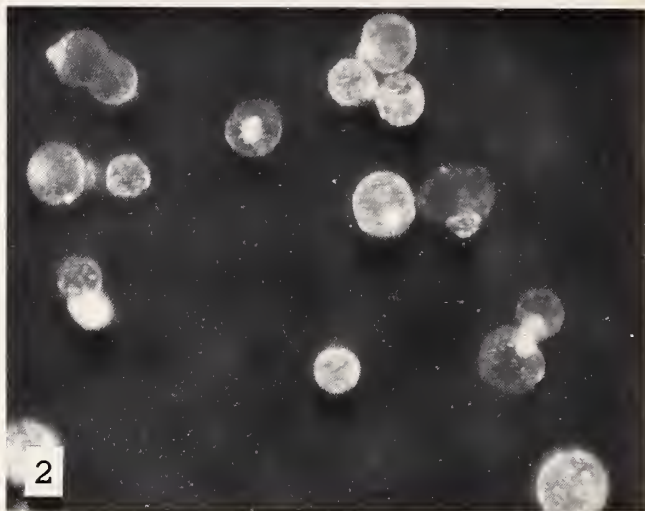


FIGURE 2.—Cells treated similarly to those shown in figure 1 and then incubated in tissue culture medium for 1 hour 7°C. at 3



FIGURE 3.—Fluorescent material protruding from a single cell.

## Summary: Antigens in Neoplastic Tissue<sup>1, 2</sup>

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**SUMMARY**—Drawn together are the contributions given in the session dealing with neoantigen expression on chemically induced tumors. An overview of the relevant problems is presented for future study.—*Natl Cancer Inst Monogr* 35: 135–139, 1972.

### OCCURRENCE OF TUMOR-ASSOCIATED ANTIGENS ON CHEMICALLY INDUCED TUMORS

Polycyclic hydrocarbon-induced tumors, particularly sarcomas, have figured largely in studies on the expression of tumor-rejection antigens on carcinogen-induced tumors (1). Other tumor types analyzed include rat mammary carcinomas and epitheliomas, and now Hellström and Hellström (2) have described the immunologic properties of mouse and rat bladder papillomas and carcinomas induced by 3-methylcholanthrene (MCA). Tumor-associated rejection antigens are also expressed on hepatomas induced by aminoazo dyes in rats (3) and by diethylnitrosamine in guinea pigs (4). Surprisingly, however, alkyl nitrosamine-induced tumors arising in other tissues have not yet been explored in depth. Tumors induced by aromatic amines, another major class of chemical carcinogens, also have yet to be analyzed in detail, the only comprehensive reports so far being on hepatomas and mammary carcinomas induced by 2-acetylaminofluorene (AAF) in rats (5, 6). Despite the broad developments in tumor immunology, therefore, the range of tumors in terms of histologic type or carcinogenic agent is still limited; this must be viewed as an important aspect of chemical carcinogenesis for future development.

#### Antigenic Variability

Although many chemically induced tumors are immunogenic, this property is not a uniform fea-

ture of all tumors. This can be illustrated by the results obtained with a range of chemically induced tumors arising in syngeneic Wistar rats (table 1). All the hepatomas induced by 4-dimethylaminobenzene (DAB) were immunogenic so that immunized rats rejected a challenge inoculum of approximately  $5 \times 10^5$  cells of the immunizing tumor. Sarcomas induced by MCA showed comparable immunogenicities, although often immunized hosts rejected whole trocar grafts of tumor. In comparison, 7 of 10 hepatomas induced by AAF had no demonstrable immunogenicity, since immunized rats failed to reject challenges with as few as  $10^3$  tumor cells. Likewise, mammary carcinomas and ear duct carcinomas induced by AAF were either weakly immunogenic or nonimmunogenic.

These studies indicate a variability of antigen expression on chemically induced tumors. They suggest that some tumors are truly deficient in tumor-associated rejection antigens. The reason for this variability, however, has yet to be defined. One hypothesis suggests that tumors lacking immunogenicity express tumor antigens, which, however, are coated by cell-surface-associated sialic acids. In this respect, however, treating cells of AAF-induced rat tumors with neuraminidase did not increase immunogenicity. Similarly, Curry and Bagshawe (7) could not induce immunogenicity in 1 nonantigenic MCA-induced murine sarcoma by neuraminidase treatment.

An alternative argument initially proposed by Prehn (8) suggested that tumor-associated antigens may be subject to immunoselective pressures so that tumors with long latent induction periods may be less immunogenic than those arising rapidly. Consistent with this hypothesis, MCA-induced sarcomas and DAB-induced hepatomas, which are almost always immunogenic, arise relatively quickly while AAF-induced tumors have longer

<sup>1</sup> Presented at Conference on Immunology of Carcinogenesis, held by the Oak Ridge National Laboratory at Gatlinburg, Tenn., May 8–11, 1972.

<sup>2</sup> These studies were made under grants from the Cancer Research Campaign and Medical Research Council.



TABLE 1.—Immunogenicities of chemically induced rat tumors

Tumor type	Carcinogen*	Number of tumors tested	Immunogenicity† of tumors—number producing resistance against challenge with:							Trocargrafts
			None	10 <sup>3</sup> cells	10 <sup>4</sup> cells	10 <sup>5</sup> cells	5 × 10 <sup>5</sup> cells	10 <sup>6</sup> cells	2 × 10 <sup>6</sup> cells	
Sarcoma	MCA	16	1	—	—	3	1	—	5	6
Hepatoma	DAB	16	0	—	—	1	11	2	—	2
Hepatoma	DENA	3	0	—	—	1	1	1	—	—
Hepatoma	AAF	10	7	—	2	1	—	—	—	—
Mammary adenocarcinoma	AAF	11	9	2	—	—	—	—	—	—
Ear duct carcinoma	AAF	3	2	—	1	—	—	—	—	—

\* Abbreviations: MCA = 3-methylcholanthrene; DAB = 4-dimethylaminoazobenzene; DENA = *N*-nitrosodiethylamine; and AAF = 2-acetylaminofluorene.

† Immunogenicity was defined by the maximum tumor cell inoculum rejected in syngeneic rats immunized either by implantation of  $\gamma$ -irradiated (15,000 R) grafts or by excision of tumor (5, 6).

latent induction periods (text-fig. 1). There are several pieces of evidence, however, indicating that this interpretation is less probable. This is emphasized by the data reported by Stutman (9) who illustrated that immunosuppression induced by antilymphocyte serum did not modify the immunogenicity of MCA-induced murine sarcomas. Furthermore, antigenicity was similarly detected in MCA-induced tumors arising *in vitro* (10) or in immunologically protective Millipore chambers (11), where immunoselection was positively excluded.

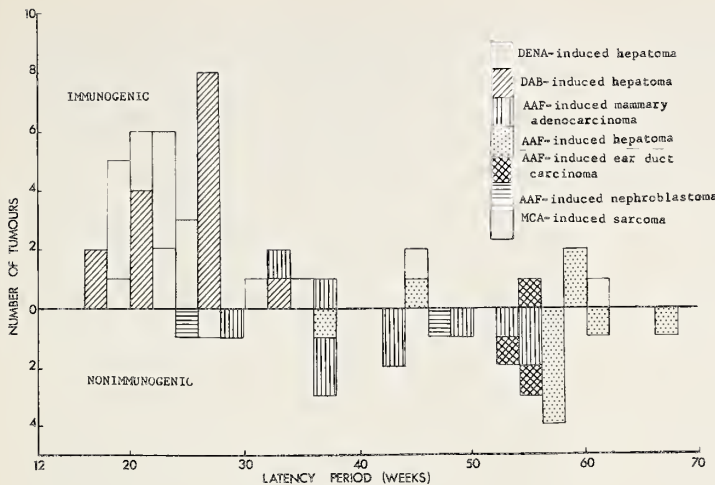
The characteristics of the antigens associated with chemically induced tumors could be accommodated by the hypothesis that chemical carcinogens produce mutation-like changes which lead to neoplastic transformation and tumor-antigen expression. If then it is accepted that both nonantigenic and antigenic tumors exist, these 2 events, transformation and neoantigen expression, need not necessarily be interdependent. This could arise, for example, if one postulates that sites of carcinogen attack in the cell genome, which lead to neoplastic transformation, do not necessarily code directly or indirectly for cell-surface proteins. Directly implied in this hypothesis is that qualitative (or quantitative) differences exist in the hepatocarcinogenic responses to AAF and DAB to account for the variability of antigen expression on hepatomas induced by these carcinogens.

### Antigen Specificity

A significant feature of the tumor-rejection antigens expressed on chemically induced tumors is

their great diversity; thus immunization with one tumor does not generally elicit resistance to other histologically identical tumors. This point was emphasized by the report of Basombrío and Prehn (17) who demonstrated unique antigenic specificities on BALB/c 3T3 cells transformed by MCA *in vivo* in Millipore chambers and is compatible with the data obtained in previous studies showing the diversity of tumor antigens on MCA-induced murine sarcomas (12). Other studies have demonstrated individual tumor-rejection antigens on MCA-induced rat sarcomas (3), DAB-induced hepatomas (3), and AAF-induced hepatomas and mammary carcinomas (5, 6). With these tumor systems, a more comprehensive and critical analysis of tumor antigen expression was obtained by use of *in vitro* methods for assaying cell-mediated and humoral antibody responses; these studies emphasized the unique characteristics of the neoantigens associated with these chemically induced tumors (3, 13). If, as already postulated, these tumor-specific neoantigens are expressions of mutation-like changes induced by chemical carcinogens, antigenic diversity may reflect the random nature of the changes leading to neoplastic transformation.

At variance with the above findings, Hellström and Hellström (2) reported the detection of cross-reacting antigens on MCA-induced rat and mouse bladder papillomas and carcinomas. In this study, lymphocytes from rats with bladder carcinomas and papillomas were cytotoxic for cultured carcinoma and papilloma cells, but not for other kinds of tumor cells or for normal rat cells. Furthermore, the appearance of primary chemically induced rat bladder papillomas could be delayed by immu-



TEXT-FIGURE 1.—Relationship between the immunogenicity of chemically induced rat tumors and latent induction period.

nization against the common bladder tumor antigens. These studies contradict the numerous reports showing the individuality of the rejection antigens associated with chemically induced neoplasms; the clearest comparable situation is the report of Lappé (14), who, however, showed specific immunogenicities on MCA-induced mouse skin papillomas and carcinomas. The question to be resolved, therefore, is the nature of the antigens associated with rat bladder tumors. These antigens appear more akin to the antigens now being detected on human tumors. One possibility is that the tumors are induced by the implanted pellet rather than by MCA, but even so, tumors induced by other inert substances such as plastic films have individually distinct antigens (15). A second and probably more likely explanation is that in addition to the individually distinct tumor-specific antigens, other neoantigens are functionally active in the bladder tumors.

This possibility is emphasized by studies on MCA-induced rat sarcomas and DAB-induced hepatomas showing that, in addition to expressing individual tumor antigens, cells of these tumors also express cross-reacting embryonic antigens detected by reaction with serum or lymphocytes from multiparous rats (3, 13). The embryonic antigens associated with these tumors appear, however, to play an insignificant role in the immune response elicited in syngeneic hosts against tumor cells. This is further emphasized by the finding that immunization against embryo cells produces only a weak and inconsistent tumor-rejection response. This anomalous finding of cell-surface, expressed anti-

gen being functionally inactive may be accounted for by studies (Baldwin and Glaves, unpublished findings) indicating that the embryonic antigens on rat hepatomas may be soluble, intracellular proteins, perhaps  $\alpha$ -fetoproteins, showing only transient expression at the cell surface during diffusion through the cell membrane. In other tumor systems, however, there is evidence that embryonic antigen may contribute more significantly to tumor-rejection reactions. For example, Coggin *et al.* (16) reported that immunization with embryonic cells could protect against hamster tumors induced by simian virus 40 or adenovirus 31 and might even interrupt viral oncogenesis. Conceivably, therefore, the immune response detected by Hellström and Hellström (2) against MCA-induced bladder tumors was directed against embryonic antigens, and this hypothesis could be verified experimentally. These studies also point to the desirability of analyzing a broad range of tumors with different organ specificities to determine the range and type of associated neoantigens as well as their immunogenic capacities.

### Characterization of Tumor-Associated Neoantigens

The investigation of the isolation and characterization of tumor-associated antigens is now at an exciting stage of development, where, after a period in which inconclusive studies were reported, precise chemical characterization of antigens is becoming possible. Two major factors have contributed to this progress: 1) the establishment of reliable ani-

mal tumor systems with well-characterized immunogenicities and 2) the introduction of reliable *in vivo* and *in vitro* antigen assays which do not rely only on tumor rejection. Furthermore, progress in studying cell-membrane-expressed tumor antigens has been accelerated by use of the methods developed for alloantigen separation.

These factors are illustrated in the report by Leonard *et al.* (17) on the use of 3 M KCl for the extraction of tumor-specific antigen from guinea pig hepatomas, the method initially used by Reisfeld *et al.* (18) for HL-A isolation. The guinea pig hepatomas studied previously were characterized immunologically, and the antigens associated with these tumors can be reproducibly analyzed by their capacity to elicit delayed-hypersensitivity reactions (19), to inhibit migration of sensitized peritoneal macrophages (20), and to stimulate lymphocyte transformation. These studies established that soluble extracts retaining tumor-specific antigen could be isolated, but the antigen yield was only 10%. Preliminary characterization of the soluble material showed that it had a molecular weight of 70,000–100,000 and it was immunogenic, protecting guinea pigs against tumor challenge.

Comparable studies with rat hepatomas and sarcomas have defined conditions under which plasma membrane fractions retaining tumor-specific antigen can be isolated, an important criterion being that nuclear damage must be minimized (21, 22). In these systems, the tumor antigen is more firmly associated with the plasma membrane, but antigen release can be effected by limited papain digestion (22). Purification of papain-solubilized antigen by ion-exchange chromatography, gradient centrifugation, and acrylamide-gel electrophoresis has yielded a single protein with a molecular weight 50,000–100,000 and with an amino acid composition closely similar to that reported for H-LA and mouse H-2 alloantigens. Although there is no evidence that tumor antigen specificity is determined by carbohydrate residues, these residues are involved in the expression of the tumor antigen at the cell surface. This was established in studies on the inactivation/release of antigen either from intact cells or isolated membrane by enzymic digestion, since  $\beta$ -glucosidase but not  $\alpha$ -glucosidase or  $\beta$ -galactosidase were active in this respect (21). The immunologic characteristics of isolated membrane and solubilized fractions from rat tumors ap-

pear different from those observed by Leonard *et al.* (17) in that these preparations do not elicit tumor-rejection reactions. This is due to an inappropriate immune response, since immunized rats consistently elicit tumor-specific antibody while the cell-mediated response is poor or negative. Moreover, *in vitro* studies indicated that serum from membrane-immunized rats was highly effective in blocking lymphocyte-mediated reactions, and the sum result of these responses was a poor level of tumor immunity. Sometimes, also, membrane immunization rendered animals unresponsive to the protective immunity elicited by intact tumor cells. These observations emphasize the need for more detailed studies on the isolation and characterization of antigens associated with a range of experimentally induced tumors and for caution in the use of isolated tumor cell fractions for clinical studies.

## CONCLUSIONS

The objective of the session was to outline progress in studies on the antigens in neoplastic tissue with the use of tumors induced by chemical carcinogens. If these systems are viewed as models of the human disease, a much broader spectrum of tumors needs to be examined. This is emphasized by the almost total lack of knowledge on the immunology of experimental tumors arising in the lung or the gastrointestinal system. Furthermore, it remains to be established whether the rat and mouse bladder tumors reported to express common organ-specific neoantigens (2) approximate more closely the human situation than often studied tumors such as polycyclic hydrocarbon-induced sarcomas.

There is little doubt that many chemically induced tumors express individually distinct antigens, and elucidation of their significance in neoplasia is an outstanding problem. A crucial question relevant to studies on the mechanisms of chemical carcinogenesis is whether neoantigen expression and neoplastic transformation are interdependent events. Since it is not possible to prove that some tumors are totally lacking in neoantigens, this question may be resolved by the antigenic analysis of tissue-cultured cells treated with carcinogen but which have not undergone neoplastic change. It is also clear that neoplastic transformation may be accompanied by a host of cell-surface changes which may result in the appearance of several types



of neoantigens. These include embryonic antigens which may be viewed as the products of re-expressed fetal genes. The significance of these antigenic changes remains to be explored.

A more practical question posed by Dr. Rausher's challenges in the opening address is: What can immunology contribute to cancer detection and treatment? This can best be explored with well-defined animal models, such as those discussed during the session, and several pertinent points were raised. Well-characterized tumor antigen is a basic requirement for immunodiagnostic techniques as illustrated by the present position on the use of carcinoembryonic antigen in the detection of carcinoma of the colon. The development of methods for antigen isolation and characterization from different tumors is therefore most timely. Although the antigen preparations so far isolated require further purification, the methodologies developed should be applicable to comparable human tumors, and so allow the isolation of membrane-expressed human tumor antigens. Undoubtedly a major objective in tumor immunology is the evaluation of immunotherapeutic procedures. Implicit in this is the need for a much broader understanding of the interaction of cellular and humoral immune parameters during tumor growth and regression, particularly with regard to the immune status of the host.

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## GENERAL DISCUSSION

**R. W. Baldwin:** My presentation was not just a summary of the papers. I hoped to bring out a few controversial points on the characterization and definition of antigens on neoplastic tissues.

To begin, Dr. Sigel wanted to make some comments in relation to these topics.

**M. M. Sigel:** The work I will describe was done in collaboration with Dr. Paul Meyers, Dr. Diana M. Lopez, and Mr. Howard T. Holden.

Immunization of chickens with Rous-associated virus (RAV) confers protection against tumors induced by Rous sarcoma virus (RSV). RAV, which does not cause morphologic cellular transformation, functions as a helper to strains of RSV which cannot complete their replication in certain cells by providing information for the production of the viral envelope. Under those conditions, the cell produces RAV and RSV progeny bearing antigenically similar envelopes. The sarcoma viruses and their helpers, some of which cause leukosis in chickens, are classified into 5 groups, A, B, C, D, and E, the distinctions being determined by the envelope antigens.

As table 1 shows, immunization with RAV-1 of subgroup A protects the birds against tumor formation after inoculation of RSV(RAV-1). The immune animals develop antibodies against the RAV-1 envelope which can neutralize the antigenically homologous RSV(RAV-1). Thus, in this kind of an experiment, it is difficult to discern the role of cellular and humoral factors in providing resistance to tumor development and growth. However, table 1 also shows that immunization with RAV-1 protects the chickens against tumor induction by SR-RSV, the Schmidt-Ruppin strain of RSV which belongs to the antigenically distinct subgroup B. In this case, prechallenge sera have no neutralizing antibodies against the challenge virus. Reciprocal experiments have shown that this type of heterologous protection could be achieved by immunization with strains of RAV of subgroups A, B, and C. The findings that cross-protection against sarcoma could be attained in the absence of circulating neutralizing antibodies against the antigenically heterologous sarcoma virus suggested that cellular immunity was being evoked by the helper viruses.

Partial support for this inference was obtained from studies on blastogenic transformation of lymphocytes from immunized birds. Such lymphocytes responded to homologous virus (of the subgroup used in immunization) as well as to heterologous virus. It has not yet been determined whether the response was elicited by a common structural antigen of the virion or a common antigen induced by the virus. Text-figure 1 illustrates the kinetics of appearance of lymphocytic sensitivity. The earliest response occurs at 4 weeks after immunization and reaches a peak at about 6 weeks. The peak resistance to tumor induction also becomes apparent at 6 weeks. This resistance persists for at least 9 months. Lymphocytes obtained at this time still demonstrate sensitivity to the antigen.

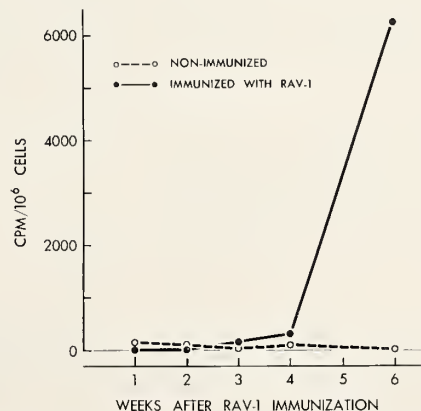
Work has begun on tumor-specific transplantation antigens (TSTA) on membranes of cells transformed by RSV. These involve cytotoxic reactions (as measured by  $^{51}\text{Cr}$

release) by sera directed against membrane antigens. These sera are obtained from hamsters bearing SR-RSV-induced sarcomas. A typical example is shown in table 2. The reaction is highly specific, since it is not exerted by normal serum and is not directed against hamster cells transformed by another virus, simian virus 40 (SV40). The MSR-5-transformed cells and LL-1 SV40-transformed cells originated from an inbred strain of hamsters. Clone 2 represents RSV-transformed cells derived from a noninbred hamster. Antigens isolated from membranes of RSV-transformed cells with the use of cysteine-activated papain or KCl have inhibited the cytotoxic reaction of the antibody. One experiment is shown in table 3. Membrane antigens from normal cells did not block the reaction. The antigen has been partially purified by gel filtration on Sephadex G-75, and further work is in progress.

**Baldwin:** We've heard from Dr. Sigel about characterization of antigens on another tumor system (Rous sarcoma). Are there any points that we should discuss about the frequency of tumor-specific antigens in any other tumor system?

**G. L. Bartlett:** It is interesting to observe "nonantigenic" tumors among tumors induced by the carcinogen 3-methylcholanthrene (MCA), which historically has been notorious for causing strongly antigenic tumors. Perhaps more important is the question about tumors developing without the action of any known or experimentally applied carcinogens, *i.e.*, "spontaneous" or naturally occurring tumors. We should direct our questions to the presence, absence, or weakness of tumor-specific antigens in those tumors. Some of my work has examined this problem. "Spontaneous" tumors arising in diffusion chambers fail to express strong TSTA. This suggests that the weakness or absence of the antigenicity of these tumors is not a function of immunoselection.

This raises another conceptual question: If naturally occurring tumors by and large lack *effective* TSTA, what then is the potential that immunosurveillance could operate



TEXT-FIGURE 1.—Incorporation of  $^3\text{H}$ -thymidine by lymphocytes stimulated by RAV-1 antigen.



TABLE 1.—Protection by RAV-1 against tumor induction by RSV(RAV-1) and SR-RSV

Immunizing virus	Challenge virus	Tumor incidence*	Presence of antibody at time of challenge to:†	
			RSV(RAV-1)	SR-RSV
RAV-1	RSV(RAV-1)	5/32 (16)	27/31 (87)	NT
None (control)	RSV(RAV-1)	33/33 (100)	0/33 (0)	NT
RAV-1	SR-RSV	13/35 (37)	29/35 (83)	0/35 (0)
None (control)	SR-RSV	33/33 (100)	0/33 (0)	0/33 (0)

\* Number with tumor/No. challenged (% positive).

† Number with antibody/No. tested (% positive). NT = not tested.

against such tumors? This might fall into the category of Dr. Lappé's question concerning when immunosurveillance may not work.

**Baldwin:** Any comments?

**P. Koldovsky:** I would like to make some additional comments on Dr. Baldwin's earlier discussion on methodology. We have to rely on more than one method to detect whether a tumor is antigenic. I would like to refer to some data published a few years ago by Klein *et al.*<sup>1</sup> These data demonstrated that practically no resistance against tumor induced by film could be detected by grafting; however, when the adoptive transfer method was used, by which enough immune cells were supplied, the tumor growth was inhibited.

Another possibility of negative results is that a tumor growing *in vivo* can already be coated by antibodies and thus be protected (enhanced). It would be interesting to look at the tumor which appears nonantigenic, after a few passages *in vitro*, to see if it becomes antigenic.

Globerson and Feldman<sup>2</sup> observed another way a tumor escaped immunity. They showed that, after a few passages *in vivo*, a benzo[a]pyrene-induced tumor was almost non-immunogenic. However, if that tumor was tested on an animal presensitized with the same primary tumor, it was still immunosensitive.

TABLE 2.—Percent release of <sup>51</sup>Cr due to serum cytotoxicity

Hamster	Cells		
	MSR-5*	Clone 2†	LL-1‡
A§	36.1	63.5	1.5
B§	18.2	68.8	1.5
Normal (control)	2.4	4.9	-1.2

\* Transformed by SR-RSV; syngeneic.

† Transformed by SR-RSV; allogeneic.

‡ Transformed by SV40; syngeneic.

§ Hamsters A and B had tumors induced by SR-RSV.

<sup>1</sup> KLEIN G, SJÖGREN HO, KLEIN E: Demonstration of host resistance against sarcomas induced by implantation of cellophane films in isologous (syngeneic) recipients. *Cancer Res* 23:84-92, 1963.

<sup>2</sup> GLOBERSON A, FELDMAN M: Antigenic specificity of benzo[a]pyrene-induced sarcomas. *J Natl Cancer Inst* 32: 1229-1243, 1964.

I would like to make one further comment directed to Dr. Sigel's presentation: The RSV family contains many strains that differ in the virus-neutralizing characteristic. However, all strains of RSV induce common TSTA. The simplest way to study the nature of TSTA is to use a non-producing system, such as the one used in mammals. A few years ago, we<sup>3</sup> showed that all the various strains of RSV that differ in neutralizing activity induce in mice tumors with cross-reacting transplantation antigen.

**J. H. Coggin:** Dr. Baldwin, it is very interesting that you do find nonantigenic, or apparently nonantigenic, tumors. But it is also continuously important to look back at the human tumors and ask, in the situations where they have been studied, how many of those are nonantigenic. Perhaps Dr. Hellström could answer that by giving us a rough percentage of those human tumors she has looked at in which she did not find autochthonous lymphocytes which were cytotoxic.

**I. Hellström:** Our data<sup>4</sup> clearly show that most patients are reactive on their tumors; 51 of 58 patients tested on their autochthonous tumor cells were reactive, while 78 of 87 patients reacted when tested on allogeneic neoplastic cells of the same type as the patients had. Our more recent, unpublished data are very similar, showing that roughly 85% of all patients have a detectable cell-mediated im-

TABLE 3.—Blocking of antibody cytotoxicity by soluble antigen extract from SR-RSV-transformed cells (MSR-5)

Target cell*	Dilution of antigen extract	Percent inhibition of <sup>51</sup> Cr release
MSR-5	Undiluted	67
	1:10	67
Clone 2	Undiluted	68
	1:10	40

\* Labeled with <sup>51</sup>Cr.

<sup>3</sup> BUBENÍK J, KOLDOVSKY P, SVOBODA J, et al: Induction of tumors in mice with the three variants of Rous sarcoma virus and studies on the immunology of these tumours. *Folia Biol (Praha)* 13:29-39, 1967.

<sup>4</sup> HELLSTRÖM I, HELLSTRÖM KE, SJÖGREN HO, et al: Demonstration of cell-mediated immunity to human neoplasms of various histological types. *Int J Cancer* 7:1-16, 1971.

munity against their tumors. We do not know whether the 15% negative findings are due to technical errors (*e.g.*, normal cells rather than tumor cells growing *in vitro*) or whether they represent a true lack of ability of the patients to react against their tumors. Other investigators<sup>5</sup> studying the same things have sometimes found reactivity at about the same level as we and sometimes a lower reactivity. Since patients with large tumor loads have less reactive lymphocytes than those with little tumor, as detected by titration of lymphocyte effects at various cell doses,<sup>6</sup> it is not surprising that the frequency of positive data reported from various laboratories can vary, less cytotoxicity being obtained if only lymphocyte:target cell ratios are used which are relatively low.

**A. J. Girardi:** I wanted to make one comment on whether the antigens are expressed or not expressed, *i.e.*, whether they are expressed but masked. In several instances, unmasking a component is necessary before it can be measured by the technique being used. It is even conceivable in the tumor that, depending on what else is turned on in the cell, a very good antigen may be present, but covered. For instance, if the cell is making some type of a coating substance (*e.g.*, collagen), that may actually cover up sites with an antigen, expressed just as in another cell. It's important to try to modify the surface to find these differences.

**R. F. Barth:** Dr. Baldwin, have you been able to produce blocking by adding purified antigen to lymphocytes and then putting these lymphocytes on target cells? Have you been able to produce blocking by adding antibody-antigen complexes to lymphocytes and then adding these lymphocytes to the target cells?

**Baldwin:** We haven't done the first experiment you mentioned. We have added tumor-bearing serum to target cells, which blocks lymphocyte-mediated cytotoxicity. Then if we go to the highly immune status, where the animals have been repeatedly immunized against the tumor, that serum will block only when put onto the target cells but not when put onto the lymphocytes.

**I. P. Witz:** I have the strong impression that several people talking about antigenicity and immunogenicity are talking about quite different things. Dr. Baldwin, could you define for us what we should consider as antigenicity—which is probably the ability of the tumor cell to react with any component of the immune system—and what we should consider as immunogenicity—which is the induction of any kind of immune response. I have the feeling that we are confusing these terms.

**Baldwin:** You've said what had to be said, Dr. Witz. Immunogenicity describes the response when the cell or component of the cell induces an immune response, but I don't think you can restrict this definition only to when a protective response is induced. If a tumor-specific response of any sort is produced, then your fraction is immunogenic, whether it is antibody or lymphocytes, or both.

<sup>5</sup> BUBENÍK J, PERLMANN P, HELMSTEIN K: Cellular and humoral immune responses to human urinary bladder carcinomas. *Int J Cancer* 5:310-319, 1970.

<sup>6</sup> HELLSTRÖM I, HELLSTRÖM KE: Manuscript submitted to *Fed Proc*, summarizing material presented at a symposium at Atlantic City, N.J., April, 1972.

Antigenicity, of course, as you say quite correctly, is present when the cell or fraction will react with one of those elements. I'm not sure that in the back of your mind you are not really questioning whether *in vitro* reactions detected in the autochthonous host reflect immunogenic or antigenic properties. Obviously, under those circumstances, the tumor cells are responding both as an immunogen and as an antigen.

**O. Stutman:** I have 3 points that I would like to make. One is regarding MCA-induced tumors *in vitro*. We have grown MCA-induced sarcomas, some highly antigenic and others nonantigenic, and their antigenicity or lack of it remains the same for long periods *in vitro*.

The second point is in regard to transformation of non-antigenic MCA-induced tumors into antigenic tumors by enzyme treatment; neuraminidase, trypsin, pronase, etc., have been consistently negative in producing antigenic changes, when antigenicity was measured by neutralization *in vivo* or by *in vitro* techniques.

The third point relates to Dr. Barth's question: I just presented at The American Association for Cancer Research in Boston evidence that, in a mammary tumor system, if you preincubate immune lymphocytes with antigen for 30 minutes and then add those lymphocytes to the target cells, they become activated and kill more effectively. You can do that preincubation in the constant presence of blocking antibody, or you can combine both together and add them to the immune lymphocytes; they will still be activated that way.

Dr. Hellström, do you think that, in bladder tumors induced by MCA pellets, MCA is required because the pellets may induce the tumors, which can explain the cross-reactivity like in tumors induced by plastic film?

**I. Hellström:** MCA is not required for induction of the bladder tumors. Papillomas of the rat bladder can be induced by insertion of pellets containing, *e.g.*, cholesterol or silastic foreign bodies, and papillomas so induced cross-react with those induced by MCA, when tests for lymphocyte-mediated tumor immunity are performed.

**Baldwin:** Dr. Hellström, if I am correct, tumors induced by plastic films do not cross-react.

**I. Hellström:** I think such tumors should be studied more, before one could categorically conclude that they do not cross-react, but as far as they have been studied, *in vivo* and *in vitro*, they have only shown evidence of individually unique tumor antigenicity.

**G. Mathé:** Dr. Hellström, have you studied a human tumor variety which is probably induced by chemical carcinogens, such as lung tumor or "professional" bladder tumor? Dr. Baldwin, how can you reconcile Dr. Hellström's data and conventional concept on antigenicity of tumors induced by chemical carcinogens?

**I. Hellström:** It is, of course, difficult to say what human tumors are chemically induced. According to some authorities on chemical carcinogenesis, chemical carcinogens are involved in the induction of some 50% of human neoplasms; if these authorities are right, we have, of course, studied many chemically induced human tumors. I suppose carcinomas of the lung and bladder are particularly likely candidates for being triggered by chemical carcinogens. We have tested too few lung carcinomas to allow any general



conclusions as to their antigenicity; however, 5 of 6 lung carcinomas we have studied cross-reacted. Bladder carcinomas in man have been tested extensively by Bubeník *et al.*<sup>7</sup> and they do indeed cross-react with each other and not with other human neoplasms. Dr. Perlmann is presenting more data on that at this Conference.

**Baldwin:** I will make one comment, because I think this is a very controversial point, and I'm sure Dr. Hellström also wants to resolve this question. Is it possible that, in your bladder tumors, you are detecting a response to embryonic antigens on the cell surface which happen to be more firmly associated with the cell membrane than in some of the other situations?

In our studies with chemically induced rat hepatomas and sarcomas, the embryonic antigen expressed at the cell surface doesn't seem to materially contribute to the tumor immune response. This may reflect the relative concentration of tumor-specific and embryonic antigens at the cell surface; also, we have evidence that the embryonic antigen is not firmly bound to the plasma membrane.

Now, Dr. Coggin has a comment, and he will also tell you that in the simian virus 40 (SV40) system, the embryonic antigen contributes to the immune response. This is really the question I want to put to Dr. Hellström: Does this reflect differences between the nature of embryonic antigen expression on various cell types?

**Coggin:** There is a lot of merit in what you say obviously, but there is a way that these embryonic antigens may play a very important role in the immune response, as you showed very well in your presentation. If embryonic antigens indeed play a role in blocking, because they are soluble from the cell *in vivo* and can coat the tumor and the lymphocytes, they may play a major role in the immune response. Embryonic antigens may offer an approach for cancer control, if their role in blocking can be confirmed and if their unique synthesis can be described.

**S. S. Tevethia:** Dr. Baldwin, will the mixture of cytoplasmic-soluble protein that induces the synthesis of antibody against embryonic antigens and sera block the lymphocytes' cytotoxicity?

**Baldwin:** No, we haven't done that experiment yet.

**Tevethia:** Will the antigen isolated on the Sephadex G-200 react with sera from multiparous rats and also from the rats that have been immunized with the irradiated tumor cells that form antibody only against the TSTA?

**Baldwin:** I just don't know. I think this really illustrates the state of development of antigen-fractionation technique. To get profiles like the one I described is very difficult, and we haven't yet managed to analyze for the distribution of the embryonic antigen in relation to the tumor-specific antigen. These are all questions waiting to be answered, but I would just add, in this respect, that the methods for antigen analysis are still really imprecise.

**Tevethia:** Do your chemically induced, nonantigenic tumors contain embryonic antigens in the absence of TSTA?

<sup>7</sup> BUBENÍK J, PERLMANN P, HELMSTEIN K, *et al.*: Immune response to urinary bladder tumours in man. *Int J Cancer* 5:39-46, 1970. BUBENÍK J, PERLMANN P, HELMSTEIN K: Cellular and humoral immune responses to human urinary bladder carcinomas. *Int J Cancer* 5:310-319, 1970.

**Baldwin:** Some do and some don't is the simple answer.

**Tevethia:** Are you suggesting then that the embryonic antigens cannot act as TSTA?

**Baldwin:** Yes, but I don't think it's a generalization that you could make.

**Tevethia:** But then there are 2 different embryonic antigens—one that can immunize and one that cannot.

**Baldwin:** No, I think it depends on their physical characteristics when they are on the cell surface.

**J. Longstreth:** Dr. Baldwin, have you tried absorbing out your hepatoma cells on another rapidly growing tissue, such as regenerating liver, to see if perhaps your fetal antigen is not just a characteristic of rapidly growing tissue?

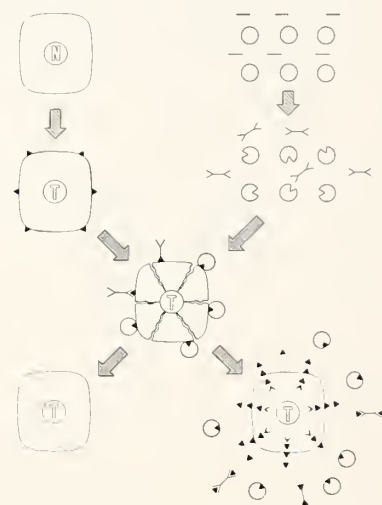
**Baldwin:** Yes, we've looked for fetal antigen on both rapidly growing liver and liver cells in culture. We don't find this antigen associated with these cell types.

**C. McKhann:** I would like to reinforce a point made briefly twice already by Dr. Weiss and by Dr. Leonard. This is concerned with surveillance and escape mechanisms (text-fig. 2).

If one assumes that one of the primitive purposes of the immune response is surveillance against aberrant cells, one can postulate that the normal cell, in becoming malignant, is more or less obliged to acquire new conformational surface properties that make it antigenic. These antigenic properties activate the surveillance response, and the aberrant cell is stamped out most of the time.

This leaves the tumor with two reasonable alternatives for escape. One is to remain malignant, but get rid of these antigenic "flags" on the surface, and essentially become

#### IMMUNE SURVEILLANCE AND ESCAPE



TEXT-FIGURE 2.—Scheme for immune surveillance and escape based on the assumption that: Many tumors must become antigenic in the process of transformation and that this is capable of arousing an effective immune response. Escape can be by *a*) decrease or complete loss of antigenic properties or *b*) massive production and export of antigen in a form that can saturate and nullify the specific immune response.



bald again—we certainly have some evidence of tumors that are not antigenic and they may have had bad antigens at one time which they discarded.

The other alternative escape mechanism is for the tumor cell to become a veritable factory of antigen. This may be antigen that is no longer tightly adherent to the cell surface but tears off easily.

Alternatively, antigen may actually be extruded from the cell surface, as was shown by Dr. Leonard, where it can saturate the system with antigen, throwing sand in the eyes of the entire surveillance mechanism.

**Sigel:** I would like to return to the point of the carcino-embryonic antigen (CEA) being responsible for some of the immune responses. Several groups of workers have shown that CEA fails to stimulate blastogenic transformation in patients' lymphocytes, even though Dr. Coggin's data showed stimulation in his animal models.

Also previous work of Drs. S. von Kleist, P. Burtin, and P. Gold did show by fluorescent microscopy the presence of CEA on cell-surface membranes.

Yet, Dr. Baldwin, you seemed to find none of it on the membranes, and all of it comes out—gushing out—from the interior of the cell. Would you kindly speculate about that?

**Baldwin:** Certainly CEA appears on the surface of the cell. I didn't have time to present the data, but serum and lymphocytes from multiparous rats are cytotoxic for tumor cells. This indicates that embryonic antigens can function as surface antigens, so that immune reactions cause cell lysis. On the contrary, the question as to whether embryonic antigens on rat tumor cells can function as immunogens is not satisfactorily resolved. I don't know whether CEA at any time can evoke an immune response in the host.

**D. A. Clark:** Dr. Gold reported circulating antibody to his CEA preparation in patients with nonmetastatic colon tumors, so that there may be some sort of immune response. CEA is immunogenic in animals, such as goats, so why not in man? Dr. Gold's inability to stimulate lymphocytes with CEA might be explained by the fact that CEA is a soluble "antigen" or by the presence of "blocking antibodies" in the patient's autologous serum included in the lymphocyte cultures.

**P. Burtin:** I would like to comment on CEA. It's not possible to make a strong correlation between human CEA and transplantation antigens, even of the embryonic type described by Dr. Baldwin. Also, I do not believe in CEA antibodies, and thus I don't think that negative results obtained by Dr. Gold in lymphocyte stimulation are due to blocking by antibodies against CEA.

**Coggin:** Dr. Girardi suggested something that may bear on the ability to obtain a blastogenic response in a patient with a colonic tumor: If this antigen is released rather readily from the tumor and coats the lymphocyte, then the lymphocytes are already coated with the antigen; to represent them with more antigen might not elicit the expected kind of response. So I think first you have to eliminate the possibility that the lymphocytes are coated with CEA. I am not sure that has been done.

**M. M. Black:** I would like to throw out an observation for comments in regards to CEA in antigen in contrast to neoantigens on tumors. Most polypoid adenomas of the

colon and most early carcinomas of the colon show a strong local reaction in terms of lymphoid and plasma cell infiltrations.

In contrast in benign teratoid lesions containing embryonal gut tissue, there is no such lymphoid cell reaction.

The gut lesion in a teratoid tumor should have CEA—yet I've never seen one that provokes a reaction. On the other hand, when a teratoid lesion shows lymphoid cell responses, it usually indicates a malignant transformation.

**H. T. Wepsic:** I have a question directed in general to people who are doing antigenic extractions. The phenomenon of tumor-associated antigens is the one I would prefer to talk about, but here we have normal antigens, tumor antigens, and perhaps embryonic antigens.

Dr. Leonard, in your system, to what extent do you have strain-2 antigens in your soluble tumor preparations? Have you done any skin grafting? Have you done any cross-blast transformation or cross-migration studies to see just how much strain-2 antigen you have in your soluble tumor antigen preparations?

And the same question can be directed to all the people who were talking about antigen-extraction procedures. Just what is the percentage of normal tissue antigens present in tumor antigen preparations?

**E. J. Leonard:** We haven't done any of those experiments. However, since about 1000  $\mu\text{g}$  of material is required for a positive skin test, or about 1000  $\mu\text{g}/\text{ml}$  culture fluid for blastogenesis or macrophage-migration inhibition (*see* text-fig. 3 in my presentation), my guess is that not more than 1% of those 1000  $\mu\text{g}$  is specific antigen.

**Stutman:** Regarding Dr. McKhann's remarks, I would like to remind you that the immune system serves other purposes, too, like dealing with infectious agents. In our heavily suppressed animals, we have to keep them as pathogen-free animals because we have to deal with infections as a major complication. I believe that is the major cause of death in the human immune-deficiencies diseases.

**R. B. Herberman:** I would like to remark on the specificity of virus-induced antigens as opposed to fetal antigens, as raised by Dr. Girardi in his Introduction. Dr. C.-C. Ting in our laboratory has demonstrated that there seem to be 2 classes of antigens which one can identify serologically. One class is specific for each type of virus induction, with specific antigens for SV40, polyoma, and MSV. Fetal antigens appear to be in a different class. Tumors induced by various agents may contain the same fetal antigens. These fetal antigens do not appear to be the same as any of the specific virus-induced antigens.

With regard to the type of *in vivo* experiments referred to by Dr. Girardi, it's very difficult to sort out this kind of thing. When one immunizes with fetuses, and then challenges with one tumor or another, it is very difficult to say that protection against the different tumors is due to the same antigen or different antigens.

**Girardi:** What we have seen is that generally there is a specificity involved in the virally induced tumors, and you can still get protection by the use of the fetal antigen. This did not imply that the SV40-induced, adenovirus-induced, or MSV-induced tumors might not also contain other antigens of various types, including fetal, that would not have participated in that initial tumor-resistance-inducing

kind of experiment. I dealt with that just in trying to answer the question that several people asked concerning fetal antigens and protection: why fetal antigens will cross-protect against so many tumors, whereas the viral tumors are specific. The answer may lie in what's turned on by the virus.

I didn't mean that the virus turns on only one thing; but from the standpoint of protection, it turns on that one spectrum of things important for specific induction of immunity.

**Herberman:** The important issue that I am raising is whether the oncogenic virus may turn on fetal antigens or actually code for TSTA. Both of these mechanisms produce transplantation antigens; yet they are two distinct mechanisms.

**Girardi:** Yes, but again, in the 10-day fetal tissue, which is active against all 3 types of tumors, instead of being specific, that kind of cell may have many specificities because normally those specificities are expressed in the fetal tissue, whereas normally you don't express an adenovirus specificity in an SV40-induced tumor. I didn't mean to overinterpret that one point.

**Herberman:** I would also like to comment on the CEA question: In some work in our laboratory involving skin testing for delayed hypersensitivity, a carcinoembryonic type of antigen has been shown in which membrane extracts of intestinal cancer, or soluble fractions from these, gave positive skin reactions. This antigen has also been found in

fetal intestinal material. In partially purified extracts, the skin-reactive antigen and the serologic CEA coexist. However, on further fractionation on acrylamide gel electrophoresis, one can separate clearly the CEA from this other CEA which causes the skin reactions. This may be one of the explanations for the differences as to whether a particular intestinal-cancer antigen causes cellular immunity or not.

**Unidentified speaker:** As someone who has come here to learn and who is still looking for consensus, I wonder if it might be appropriate, as this is the last question, to ask chairmen of subsequent sessions to see whether some consensus couldn't be brought together on 2 points. One is the nature of blocking factors. There seem to be people here who have information relating to the mechanism of action of blocking factor—whether it acts with the target cells or the lymphocytes, or where it acts. I would ask the Conference organizers to bring some of these people together to provide the consensus that we were asked to provide on that point.

A second point is we have people who are interested in the cellular aspect of cytotoxicity. We are all talking about lymphocyte cytotoxicity, but there is a lot of evidence in the literature now that the lymphocyte itself may not be the major mover in this thing, but the macrophages, or even polymorphs, may be involved; can we have some kind of consensus on this point, perhaps to serve as the direction for further study, if not establish some facts?

**Baldwin:** This is the subject of the meeting in Session 5.

## **SESSION 4**

### **Modification of Immunity and Carcinogenesis**

**Chairmen: Jan Stjernswärd and Nechama Haran-Ghera**





# Introduction: Modification of Immunity and Carcinogenesis<sup>1, 2</sup>

Jan Stjernswärd, *Department of Tumor Biology, Karolinska Institutet and Radiumhemmet, Karolinska Sjukhuset, Stockholm 60, Sweden*

**SUMMARY**—Enough clinical evidence seems to support immunologic surveillance as a legitimate concept. Chronic immunosuppression may be related to an increased frequency of solid tumors, and chronic immunostimulation, especially in an already immunosuppressed host, may be related to an increased frequency of lymphoid tumors. Models now exist in man for constructive and fruitful analyses, both quantitative and qualitative, of the degree of importance of the immune responses with respect to tumor growth.—*Natl Cancer Inst Monogr* 35: 149–156, 1972.

WHEN REVIEWING the literature for the introduction to this session, I became hesitant: Why bother you with another review (1–14), boiling the same old soup of things you knew just as well as, if not possibly much better, than I? But then I discovered that some constructive models in man could be presented, and the same, original data, which have been repeatedly quoted as favoring the existence of an immunologic surveillance mechanism, could be interpreted in exactly the opposite way. It is still open to question to what degree postulated immunologic surveillance mechanisms function clinically in man. However, certain data could be construed, as yet only indirectly, to favor a relationship between immunocompetence and malignancy in man. These would include the following:

- 1) Association between variations in tumor incidence with age and age-correlated changes in immunologic reactivity.
- 2) Depression of thymus-dependent immunity—whether genetic or iatrogenic—correlated with increased tumor incidence.
- 3) Higher frequency of tumors, especially in early childhood, than later clinically manifested.

- 4) Frequent elimination of minimal residual tumor dose.
- 5) An association between chronic antigenic stimulation and increased frequency of lymphoid tumors.

Solid clinical data demonstrate that tumors once developed still are not completely autonomous in their growth (15). The following evidence indicates the existence of tumor-limiting host factors: spontaneous regression, regression of metastasis after resection of primary tumor, regression of tumor despite “nonkilling” doses of chemotherapy, reappearance of metastasis after a long latency period, frequent failure of circulating tumor cells to form tumors, histologic evidence of immunologic reactions to tumors, and regression of tumors surrounded by a local, delayed-hypersensitivity-type reaction (16). It would be naive to think that all these observations could be explained merely by host immunity. Doubling time of tumor cells, endocrine factors, outgrowths of vascular supply, etc. could by themselves explain some observations, but host immunity cannot be excluded as a factor.

The existence of host immunity to autochthonous tumor is well established (1, 8, 10, 17–21). The question now is: Of what importance may the tumor-limiting responses be? Before one searches for relevant situations in which this could be tested, it may be worthwhile to look at data indicating

<sup>1</sup> Presented at Conference on Immunology of Carcinogenesis, held by the Oak Ridge National Laboratory at Gatlinburg, Tenn., May 8–11, 1972.

<sup>2</sup> Supported by the Swedish Cancer Society, King Gustaf V's Jubilee Fund, Agnes and Adolf Wallgren's Fund, and the Karolinska Institutet Fund.

a lack of autonomy of tumors and, thus, the existence of tumor-limiting factors in the cancer patient.

### **"NONCLINICALLY" MANIFESTED TUMORS**

At both ends of life, in early childhood and old age, there seems to be a much higher incidence of tumors found at autopsy than manifested clinically. Autopsy studies on infants who died of diseases other than tumors have shown that neuroblastomas occur in infants up to the age of 3 months at 40 times the clinically expected rate of appearance (22). Thus, for every clinically manifested tumor, there are 40 newborn infants in which the neuroblastoma will either spontaneously disappear or develop into a benign tumor (23).

Data on the elderly may indicate that every second person dies with a tumor, though not of it. At routine autopsy of 11,098 patients, representing 60% of the patients dying in one large Swedish city during a 9-year period and mainly comprising persons dying in old-age homes and mental hospitals, but also comprising some in ordinary hospitals, 44% (4,895 patients) had 5,523 primary tumors; 12% had 2 or more tumors. The dominating tumors were carcinomas. Tumors were clinically unknown in 26% of the patients (24). This example is ample evidence that tumors can be detected at routine autopsy in certain tissues with a frequency far exceeding the incidence of clinically apparent neoplasms in the patient. An analogous, older example of this is the finding with the prostate gland, in which microcarcinomas were discovered in up to 30% or more of these glands examined at autopsy in males >80 years of age who died of unrelated causes (25).

The peak incidence of cancer at early childhood and at old age is one of the absolute facts in man (26). This age-specific incidence of cancer may be related not only to accumulation of somatic mutations and carcinogenic insults (27) but also to possible variations, with age, in the immune status in man (2, 4, 28-31). However, experimental evidence exists, demonstrating a direct correlation between change in immune status with age and ability to reject small, graded doses of antigenic tumor cells (29). Probably both mechanisms, accumulation of carcinogenic insults, functioning at the induction stage, and decrease of immunologic defense mechanisms, functioning at the selection stage, synergistically favor tumor de-

velopment, at least with regard to solid tumors. Again, we must be aware of the possibility of differing pathways for solid tumors as opposed to lymphoid tumors and thus avoid generalization.

### **MODELS IN MAN TO TEST IMPORTANCE OF TUMOR-HOST IMMUNITY**

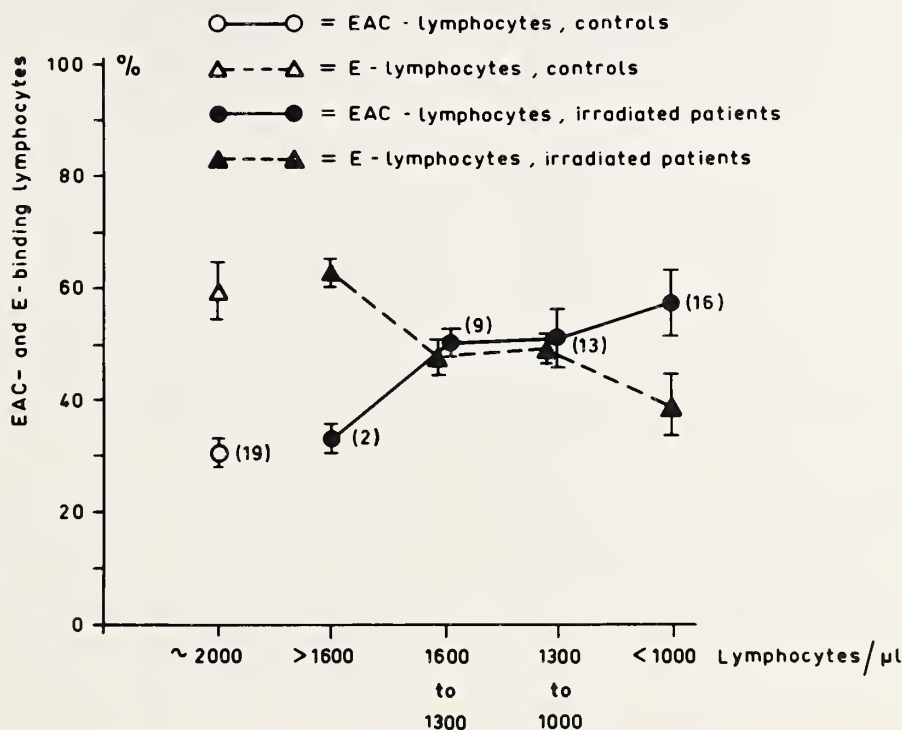
The iatrogenically induced interferences with immunologic responsiveness in man offer an ideal opportunity to test quantitatively and qualitatively whether, and to what degree, postulated immunologic surveillance mechanisms in man play a clinically important role. There are 2 distinguishable stages in carcinogenesis: 1) induction of the neoplastic cells and 2) selection of the already induced neoplastic cells. Most agents used in man, like radiation or cytotoxic drugs, can affect both stages, but we will discuss only the last of the 2 stages.

The effect of induced immunosuppression on tumor incidence was studied in kidney-transplant patients without known tumors (17, 32). A relevant study could be conducted on known cancer patients from whom the primary tumor had been removed and who, as judged by conventional diagnostic methods, had not been found to have any detectable tumor left (33). Statistical data on later metastases, however, show that a certain number must have had a minimal residual tumor dose at this time. Iatrogenically induced immunosuppression on such a group would allow an analysis of the role, if any, that interference with host immunity plays in regulating tumor growth in the patient. In fact, a strictly randomized selection of cancer patients prospectively exists where a long-lasting immunosuppression in one group can be compared with a temporary immunosuppression in another group at surgery. Patients with early mammary carcinomas irradiated postoperatively offer this ideal model system.

Irradiation to a limited area, which includes the thymus, such as routine postoperative irradiation in early mammary carcinoma to paravertebral lymph nodes and supraclavicular nodes, will cause lymphopenia of more than 1 year's duration. The irradiation-induced lymphopenia is due mainly to an elimination of the thymus-dependent, circulating T-lymphocytes, accompanied by a relative increase in the bone-marrow-dependent B-lymphocytes (33). With the increase of lymphopenia, there seems to be a proportional decrease of the T-



# Proportion of EAC- and E-binding lymphocytes in peripheral blood as related to irradiation induced lymphopenia.



TEXT-FIGURE 1.—Proportion of B- and T-lymphocytes in peripheral blood from 59 individuals, plotted for 40 patients to degree of irradiation-induced lymphopenia. The number of individuals for each observation is given in parentheses (33). Values are expressed as mean  $\pm$  SD.

lymphocytes (text-fig. 1). In normal, healthy controls, the proportion of T-lymphocytes, *i.e.*, those able to spontaneously form rosettes with sheep erythrocytes, was 60%. Thus in 1 mm<sup>3</sup> of blood in a nonirradiated patient, there will be 1,200 T-cells that the tumor cell(s) would meet. In a patient with mammary carcinoma, immediately after postoperative irradiation inducing a not unusual lymphopenia of only 500 white blood cells, the circulating T-cells will decrease to 45%. The total number of T-cells that the tumor cell would meet in 1 mm<sup>3</sup> of blood would then be 225.

The lymphopenia is of the same order of magnitude as that induced by extracorporeal irradiation known to prolong survival of antigenic transplants (34). Looking in the "answer book" to see whether the frequency of distant metastases possibly is increased in the irradiated group, as could be postu-

lated if the thymus-dependent immunity has a tumor-inhibiting effect (2, 35), we find 3 independent, randomized trials on patients who, after surgery, received either radiotherapy or no radiotherapy. These studies show that the incidence of distant metastases in the irradiated group is higher than that in the nonirradiated group (table 1).

Whether a direct correlation exists between the resultant shift in proportion of B- and T-cells in irradiation-induced lymphopenia and the later occurring distant metastases is now open to test. The look in the "answer book" above indicates this may be a relevant question to follow up. Patients with early mammary carcinoma could thus offer the ideal model to analyze quantitatively and qualitatively the degree of effect, if any, of postulated immunologic surveillance mechanisms.

The time lapse between the removal of the

TABLE 1.—Early distant metastases in postoperatively irradiated patients with early mammary carcinoma as compared with nonirradiated patients, in 3 randomized trials\*

Studies	Time following primary treatment				Criteria	Reference
	First year		Second year			
	Percent	Metastases/total No. patients	Percent	Metastases/total No. patients		
Paterson and Russell, 1959						
Radical + radiotherapy	5.0	16/327	6.1	19/311	Liver metastases	(69)
Radical	3.3	13/393	2.9	11/380		
Bruce, 1971						
Simple + radiotherapy	6.3	12/191	8.6	15/174	Mortality	(70)
Radical	3.4	7/204	4.4	7/158		
Höst, 1972						
Radical + radiotherapy	8.3	9/109	12.0	12/100	Distant metastases, stage II	(71)
Radical	0	0/92	16.0	15/92		

\* Clinical data motivating further studies on whether routine, indiscriminate use of postoperative radiotherapy in early mammary carcinoma may result in higher numbers and/or earlier appearance of distant metastases. A prospectively randomized trial of radiotherapy after simple mastectomy of >1,000 patients indicates increased frequency of distant metastases in irradiated group. After some years, this trial may definitely answer the question above. (Baum M, Edward MH, Brinkley D: Personal communication.)

primary tumor and the appearance of a recognizable recurrence varies greatly, from a few months to >30 years. When this interval is short, probably tumor cells are not disseminated in the body on removal of the primary tumor but have already reached the distant organ in which they later appear.

A valid form of immunotherapy for the interval between the removal of the localized primary tumor and the manifestation of a distant metastases is greatly needed. Therapy-induced lymphopenia with a resultant shift in the proportion of circulating B- and T-cells is an important consideration, not only in the analysis *in vitro* by various methods of the evidence for tumor-associated, tumor-distinctive immunity but also specifically in the attempt to relate this meaningfully to the *in vivo* situation. Changes not only at the tumor cell level but also in the formerly ignored lymphoid test cells are important.

Patients with immunologic deficiencies are a realistic model, leading to important conclusions (36–38). For analysis of the relationship between immunologic reaction and malignancy, I view the cancer patient, during the interval between removal of the primary tumor and later distant metastasis, as one of the best models providing critical direction for basic laboratory inquiry.

## OTHER MODELS

Direct evidence in man now exists that an increased lymphoid infiltration around small tumor

nodules in the skin, induced by a delayed-hypersensitivity reaction to a chemical allergen, may result in regression of solid tumors, such as metastases of mammary carcinoma (39, 40). A decreased ability for delayed-hypersensitivity reaction within the irradiated area of the chest wall may occur. Local recurrence sometimes can spread within the strictly confined limits of the irradiated area, and further testing is required to determine whether any relationship exists at all between the decreased ability for delayed-hypersensitivity reaction within the irradiated skin area and the pattern of local recurrence (41, 42).

The potential clinical significance of the role of the immune system providing defense against cancer is indicated by the finding, in kidney-transplant patients, that widely disseminated malignancy was completely eliminated on cessation of the immunosuppressive therapy (43, 44).

## CHRONIC IMMUNOSUPPRESSION— IMMUNOSTIMULATION

The increased incidence of solid tumors in patients receiving immunosuppressive regimens for kidney transplants confirms the earlier postulate (35) that this would be the case if immune surveillance is of clinical importance. Of 37 *de novo* tumors observed in a little over 3,000 kidney-transplant patients, 21 were carcinomas and all were primary tumors. Sixteen were mesenchymal,

and most of these were reticulum cell sarcomas (77). However, chronic immunostimulation, rather than chronic immunosuppression, may be of etiologic significance in the frequency of this type of tumor. The incidence of tumor, around 10/1,000 persons in the kidney-transplant groups, is far above that seen in an age-adjusted, normal population in which the corresponding figure was about 6/100,000 persons and <1/100,000 persons for reticulum cell sarcomas. Of 24 mesenchymal tumors which arose *de novo* in patients with organ homografts, 11 involved the brain (45). This fact opens speculation on the importance of possible immunologically privileged sites. The frequent appearance, in Burkitt's lymphoma, for example, of central nervous system relapse as the first and occasionally only sign of recurrent tumor may support the concept that the brain is an immunologically privileged site (46-48). The blood-brain barrier may be not only an immunologic but also a pharmacologic barrier which may be relevant in both examples.

Valuable information toward further understanding the mechanism may be gained by the analysis of the cause of the varying frequency with which neoplasms are observed. Even within the same treatment center, frequency variations exist, depending on the type of immunosuppressive regimen administered. Thus azathiopurine and prednisone resulted in 15 tumors in 195 patients, while these drugs plus antilymphocytic serum (ALS) so far have caused no tumors in 111 patients in the same center. A shorter observation period in the latter group cannot alone explain the difference. In the group in which tumors developed, 45 patients had been thymectomized. Of the 15 tumors, 13 were carcinomas and 2 were lymphoid tumors; 4 of the former and 1 of the latter developed in thymectomized patients (32).

The difference may be due to ALS. A reduced incidence of X-ray-induced leukemia after treatment with ALS has been observed in an experimental system (49). A speculative explanation is that ALS eliminates the neoplastically transformed lymphoid cells. However, the low frequency of lymphoid tumors contradicts this explanation.

In contrast, nonirradiated, normal hosts with ordinary numbers of lymphoid cells who were given injections of the virus and thereafter treated with ALS had significantly increased incidences of lymphoid tumors (50).

Thus the same agent, ALS, either reduces or

increases the tumor incidence, probably depending on whether the host was previously immunosuppressed. Not only elimination of neoplastically transformed lymphoid cells by ALS in the first case but also depletion by previous irradiation of target cells for a later neoplastic transformation may affect the availability of target cells to be transformed by a latent leukemogenic virus activated by, *e.g.*, chemical carcinogens (51), irradiation (6, 52), or chronic antigenic stimulation (53). Once the neoplastic transformation is induced, disturbances of immunologic surveillance mechanisms may be critical to the ability of the cells to survive and replicate (54).

Chronic immunostimulation may be etiologically significant for lymphoid tumors. The increased tumor incidence in children with immunodeficiency has been taken as supportive evidence that immunosuppression is important in malignancy. Very easily, however, this idea may be reversed to indicate that chronic immunostimulation is more important. With few exceptions, all the tumors found in the chronically immunosuppressed children are lymphoid. These patients have repeated, severe bacteria and virus infections which usually cause their deaths.

In the immunosuppressed kidney-transplant patient, a dualistic situation exists with chronic immunosuppression and immunostimulation. An analysis of the data regarding the various types of tumors, carcinomas or lymphoid tumors, arising in relation to given regimens and in relation to the degree of histoincompatibility of the antigenic kidney transplant may lead to fruitful information.

Under the influence of immunosuppression, persistent antigenic stimulation may provide the basis for cell transformation in the development of lymphoid tumors. Strong experimental data support this concept (55-57), and clinical data demonstrate an increased frequency of reticulum cell sarcomas in patients chronically immunosuppressed as well as stimulated (3, 58, 59).

Persistent immunologic stimulation by holoendemic malaria, providing a maximal and persistent stimulus to the lymphoreticular system of the young host, has been taken as a factor in the oncogenesis of Burkitt's tumor (60). Burkitt's tumor cells closely resemble lymphoreticular elements activated by an antigenic stimulus. Latent viruses, whether oncogenic or epiphenomic passengers, may



be activated immunologically (53). Hodgkin's disease, lymphogranulomatosis maligna, is similar to the graft-versus-host type of reaction in experimental systems (67). Chronic immunostimulation may be related to the development of lymphoid tumors but not necessarily to solid tumors. Therefore, we should avoid the common pitfall of not separating the 2 types of tumors and thus invalidating a correlation between certain parameters by putting the wrong tumor group in the wrong context.

## CLINICAL REALITY AND NEEDED REALISM WITH ANIMAL MODELS

When actively manipulating the immune system by immunotherapy, we are again faced with the problematic dichotomy of the immune responses. However thoroughly we analyze the various parameters of nonspecific stimulation of host resistance to tumors in experimental systems, we may never, through the experimental systems, gain relevant information applicable to man. If the timing of the adjuvant is as critical as indicated by most experimental systems, many of these experiments will not present clinically relevant models.

In most experimental animal systems, the timing of the adjuvant with respect to the later antigenic tumor-cell transplants seems critical indeed for whether we will have tumor-inhibiting immunity or facilitation (62). Besides factors such as route of administration, dose, type of tumors, and basic immune status of the host at the time of adjuvant administration, the problem of timing will also have to be solved in man. When do we know whether we have, or do not have, minimal residual tumor dose? Prophylactic administration is not realistic. The realistic time would thus be at the time of minimal, residual, unknown tumor cells in the patient. If so, the only experimental models valid are those in which bacille Calmette-Guérin (BCG) is given to a host with a tumor already present, or given to a host after removal of its tumor, either simultaneously with or after tumor test transplant.

The host's immune status when receiving BCG will also be important. Tumor is probably more likely to be inhibited by immunostimulation in an immunosuppressed host than in a host with normal immunologic reactivity. That mammary carcinoma patients receiving radiotherapy will have

a long-lasting T-cell lymphopenia, seen against the background of the indicated increase of earlier appearing distant metastases, offers this group as a good model for immunotherapy of solid tumors.

Even within the same host system, the timing is a critical, determining factor in rejection of the tumor (62). Thus if the timing must remain relatively uncontrolled, should we either abolish as unrealistic the idea of nonspecific boosting or conclude that time is now mature to test every type of tumor in man on its own value? The latter, if done critically, selecting the right groups of patients, would be my preference as a realistic conclusion.

Should such trials be done, it is mandatory to monitor objective test parameters *in vitro* and *in vivo*, so as to be able to critically analyze the results. Selective stimulation of T-lymphocytes without stimulation of humoral blocking factors will be an obvious goal. Data presented in this session will indicate that thymus-derived cells are the site of adjuvant action (63).

Emerging data indicate that boosting of immunity in immunosuppressed patients with leukemias (acute lymphocytic and acute myelogenous), after eradication of the main tumor bulk by chemotherapy and after administration of allogeneic tumor cells as well as BCG, will prolong remission time, or even survival time, in the group treated by immunotherapy, but not in the group receiving only chemotherapy (64, 65).

A theoretically logical approach for immunologic intervention in those cancer patients who really represent a situation in which postulated host immunity has failed, or has been outflanked, would be to increase or, better yet, to change the antigenicity of the malignant cells. Experimental data demonstrate that immunogenicity can be augmented (66, 67). Clinical data, although only indicative, may already support this important concept as a realistic approach in the cancer patient (68).

I hope this may serve as a conceptual framework for the data presented later in this session in the analysis of what relevant data will emerge to elucidate a responsible mechanism for future clinical manipulation. The primary question now is not the existence of immunologic surveillance but, rather, the relative degree and importance clinically of host immunity, and how it can be explored in man.

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# **Nonspecific Stimulation and Modulation of the Immune Response and of States of Resistance by the Methanol-Extraction Residue Fraction of Tubercle Bacilli<sup>1,2</sup>**

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**SUMMARY**—The salient properties are summarized of the methanol-extraction residue (MER) fraction of tubercle bacilli as a modulator of immunologic responsiveness. Detailed descriptions of the activities of MER are found in the references cited, as are discussion and speculation concerning possible mechanisms of action.—*Natl Cancer Inst Monogr* 35: 157–171, 1972.

A VARIETY of microbial and other substances can impinge on immunologic function nonspecifically. The earliest recorded examples of this were the altered reactivity to antigens introduced into tuberculous lesions and the ability of mycobacteria to act as immunologic adjuvants (1). Until recently, however, the potentiation and redirection of immunologic reactivity, which can be achieved by introduction of specific antigens together with nonspecific adjuvants, or shortly after exposure to the adjuvants, have been considered largely as a side

issue of modern immunology, albeit one of some practical interest. For decades, nonspecific exaggeration of immune responses has been a standard procedure of experimental and clinical immunology. It has been accepted for some time that some adjuvants may magnify certain immunologic reactions, occasionally direct these reactions preferentially toward one or another of the different humoral and cellular components making up the immune response to most antigens, and possess the quality of “adjuvanticity,” which leads an antigen to elicit an active immune response instead of specific tolerance or paralysis (2). Nonetheless, the properties and behavior of conventional adjuvants and adjuvanticity agents have been viewed as ancillary rather than essential to immunologic phenomena, and the axis of immunology has been drawn in two-dimensional terms: the structure of specific antigens and haptens and the qualities of immunogenicity deriving from their biochemical and biophysical nature, on the one hand, and the genetic, morphologic, and physiologic properties of the tissues making up the immunologic apparatus of vertebrate animals on the other.

However, a third and basic dimension to this formulation of immunologic reactivity should be added: the modulating effect on immune processes by families of substances which in all likelihood are not only ubiquitous in nature but also in their participation in the interplay between molecular entities carrying unrelated alien determinants and the animals with which they come into

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<sup>2</sup> Work on the methanol-extraction residue (MER) during the past 15 years was made possible by many private and governmental agencies and by individual donors. The specific sources of support for the different aspects of these studies are acknowledged in each research communication. I do, however, wish to cite the major funds that have enabled us to study MER more extensively during the past 2 years and to express my gratitude for this aid: Public Health Service research contracts NIH-71-2127 and NIH-70-2208 from the National Cancer Institute; Concern Foundation of Los Angeles; Leukemia Research Foundation of Chicago; New York Cancer Research Institute, Inc.; West Coast Physicians Division of the American Friends of the Hebrew University; Mr. and Mrs. Frank Lautenberg; Mr. and Mrs. Henry Taub; Mr. and Mrs. Joseph Taub; and Mr. and Mrs. Laurence Tisch.

<sup>3</sup> I acknowledge respectfully and affectionately the concern of Dr. and Mrs. Wilbur Schwartz with the progress of the work and with its financial backing. I also extend warm thanks to Mrs. J. Weiss for her help in preparing this manuscript.

contact. These modulating agents may often be antigenic, but the present report focuses on the involvement of these agents in immunologic responses to antigens with which they apparently share no common determinants.<sup>4</sup> Because the activities of such agents are undoubtedly fundamental to immunologic recognition and intimately involved in antigen–animal relationships (3), the classical terms “adjuvant” or “adjuvanticity agent,” which connote much more restricted effects, are inappropriate. Moreover, the mechanisms of immunologic modulation in the broadest sense would appear clearly to go beyond the effects of antigen depot, local accumulation of lymphoid tissue elements, and chemical modification of antigen structure, which have been generally thought to be the major modes of action of classical adjuvants added intentionally and artifactually to a given immunologic system (3).

It is suggested then that agents not participating in the specific antigenic behavior of given immunogens nonetheless define, under the conditions of nature, the extent and type of the immunologic response an animal will mount against them. It is also suggested that many physiologically vital immunologic reactions—which might well be termed “physiologic immunology”—are minimal, although mandatory for normal physiologic functioning, and may depend largely on extrinsic modulation for their expression. Possible examples of this physiologic immunology, aside from resistance to microbial and neoplastic parasites, are: the facilitation of the implantation of fertilized ova and perhaps the recognition of sperm cells, and some of the processes of parturition; the recognition and removal of effete autochthonous components, metabolic by-products, and alien materials other than microorganisms; participation in the processes of control governing differentiation and organ and tissue architecture and spacing; maintenance of the tone of smooth muscle and vascular endothelium, monitored perhaps by pharmacologic mediators, which, in turn, are released by ongoing antigen–antibody interactions; and many other.

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<sup>4</sup> Perhaps all or many biologic macromolecules, or macromolecular aggregates, carry overlapping specificities. If this should prove correct, the definitions of “alien” and “related” may have to be rephrased in a more precise and restrictive context, but the strong possibility remains of the modulating activity on immune phenomena of substances apart from their own specific antigenic qualities.

It may thus be argued that adaptation to accept an appreciable number of microbial moieties and products, as well as other environmental materials, as modulators of immunologic reactivity represents a necessary phylogenetic event in the development of the vertebrates and that quintessential characteristics and limitations of the vertebrate immunologic apparatus can be understood only in light of the pervasive involvement of nonspecific modulators as costimuli with specific antigens.

For that matter, nonspecific immunologic modulation may not be limited to the vertebrate kingdom. There is evidence that the role of nonspecific immunologic modulators as determinants of the capacity and form of immunologic responsiveness may be especially prominent where such responsiveness is inherently low or immature or where the immunogenic stimulus is marginal. It is theoretically difficult to assume that the ability to synthesize molecules of the complexity of the vertebrate immunoglobulins and to make cells with membrane characteristics akin in specificity to those of antibodies originated *de novo* at the level of the primitive vertebrate fish. Some provocative information indeed points to the occurrence in invertebrates of responses biologically similar to true immune reactions in vertebrates, and there have been reports of the production in invertebrate species of molecules having immune function and chemical properties suggestive of possible Ig precursors.

It is likely, however, that at least some invertebrate immunologic mechanisms are poorly developed and “weak” (though perhaps not inefficient in terms of the survival demands placed on them!) when compared to those immune reactions of vertebrates of which they are the nearest counterparts. Nonspecific modulators may thus be of special importance in the activation of lower animals to foreign determinants.<sup>5</sup>

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<sup>5</sup> It would be worthwhile to search for modulators whose activity may be limited to, or especially pronounced in, the immune-like responses of invertebrates. Special attention should be given in this quest to derivatives of microorganisms involved intimately with invertebrate animals as obligatory or frequently facultative symbionts. It may also be profitable to explore further the possibility that the absence, or at least infrequency, of true neoplasia among invertebrates reflects not a phylogenetic impossibility of neoplastic growth, but rather the ability to bring to bear highly efficient, and perhaps modulator-dependent, mechanisms for destroying the neoplastic variants as they arise.



Thus these considerations, although many of them are still largely theoretical, suggest that non-specific modulators of immune and immune-like responsiveness play a very broad and primary role in the recognition of, and reactions against, alien molecular configurations. The term "modulator" is suggested as the most suitable and inclusive description of any agent which can qualify quantitatively or qualitatively the immune response against other substances (the specific antigen), by any mechanism other than classical immunologic cross-reactivity. This definition subsumes materials referred to elsewhere as "adjuvants" and "adjuvanticity" substances, and leaves open for analysis in each instance the modes of action.

The present paper outlines the properties and activities of one modulator of immunologic responsiveness and of states of resistance, a methanol-extraction residue (MER) of tubercle bacilli. Although MER has been found in our laboratories and by other investigators to be exceedingly broad and pronounced in its nonspecific modulating and stimulatory abilities, it is emphasized that we have viewed it as a *model*. Because of the broad activities of MER and especially because of its marked ability to elevate resistance against syngeneic and autochthonous neoplastic cells, a systematic study was undertaken some years ago of the breadth of its biologic behavior, despite the fact that MER is a crude, uncharacterized, microbial fraction. There is no reason to believe, however, that MER is the most effective or otherwise most desirable non-specific enlarger and qualifier of resistance and immunologic responsiveness which exists in nature or which may be developed; the findings with MER summarized herein primarily present a model of the extent of the potentialities of substances classified as nonspecific modulators.

## MER FRACTION OF TUBERCLE BACILLI

### General Properties

As described in a recent review on MER (4), the nonspecific activities of MER were brought to our attention accidentally: During an epidemic of pasteurellosis in a guinea pig colony in England in the winter of 1956-57, animals which had been immunized with MER against later infection with virulent tubercle bacilli were surprisingly found to be uniquely resistant also to the presumably unrelated pasteurella invader.

Subsequent studies on MER were largely in two directions: 1) determination in several animal species of the range and nature of its protective effects against microbial and neoplastic challenge; and 2) analysis of its ability to enlarge and modulate immunologic responses to known antigens. In many of these systems, MER proved more potent than living bacille Calmette-Guérin (BCG) and other mycobacterial entities and was active under different experimental parameters. Possibly treatment of MER with hot methanol and the other procedures in its production have "exposed" molecular entities of the bacterium which do not otherwise come readily into biologic play (5).

Both virulent and avirulent human and bovine strains of tubercle bacilli can be used as a source of MER, but most MER with which we have worked has been from lots made by us from the BCG-Phipps strain in our former laboratories at the University of California at Berkeley. Recently, Merck, Sharp & Dohme Research Laboratories, under contract with the National Cancer Institute, prepared a large batch of MER from this strain. This lot has been milled finely after the usual preparation, sterilized with ethylene oxide, and formulated in vials in a stabilizing agent. The resulting fine suspension is considerably more stable than the crude aqueous suspensions previously made by hand-grinding the dry MER powder with mortar and pestle and then dispersing it further in a Vir-Tis high-speed homogenizer, and has proved highly active in several test systems.

The preparation of MER has been detailed in (4, 6).

The general known physical and biologic properties of MER are summarized in chart 1.

The chemical nature of the active fraction(s) is still unknown. A lipopolysaccharide moiety might be involved, perhaps related to the mycolic acid-glycopeptide compounds which possess classic adjuvant activity (7-9), but there is no direct evidence for such a possibility. It is stressed that the biologic properties of MER are both qualitatively and quantitatively very different in many particulars from those of the gram-negative lipopolysaccharide materials and other defined nonspecific modulators; if lipopolysaccharide moieties play a role in MER activity, they must possess unique properties or function in conjunction with other molecular entities.

Dr. M. Tischler, Wesleyan University, recently



CHART 1.—Known physical and general biologic properties of unpurified MER\*

#### PHYSICAL PROPERTIES:

Particulate, pale-yellow, nonviable powder, largely insoluble in water and the usual organic solvents, consisting of lightly acid-fast microbial debris and fragments. Fine dry milling does not destroy activity.

Highly stable when stored in the cold for prolonged periods, dry, and in frozen crude aqueous suspensions.

Stable to heating in neutral aqueous suspensions, at up to 70°C for 10–15 minutes, and perhaps at higher temperatures for longer periods.

Stable to sterilization with ethylene oxide.

#### GENERAL BIOLOGIC PROPERTIES:

Induces local granulomas which may persist for long periods, but, at quantities usually given for optimum activity, induces in all species local lesions that are less severe than those usually induced with living BCG.

Does not induce distal lesions and is systemically well tolerated at optimally effective dosages.

Induces tuberculin hypersensitivity in guinea pigs and in at least some humans, but to a considerably less extent than living BCG.

Is not pyrogenic.

Does not appear to induce negative phases of antimicrobial resistance, regardless of treatment–challenge intervals.

Optimum amounts are low (usually <2 mg) in different species and similar in different protection and immunologic responsiveness test systems; body weight–dose relationships do not appear to exist, at least among the several species tested.

Is effective when given by different routes and often for prolonged periods of time.

Standardly made preparations are almost always active in the various test systems.

\* For details, see (4).

initiated systematic studies directed toward the isolation and characterization of active components of MER. However, previous tentative attempts in this direction have given ambiguous results: Extracts obtained by various mixed organic solvent extractions had only sporadic activity in different systems (several preparations *were* highly, though not reproducibly, active), whereas the insoluble residues usually retained various nonspecific biologic activities as long as they remained particu-

late. Complete disruption of the particulate state of the residues always destroyed biologic activity as well (4, 10). Thus at least some of the nonspecific properties of MER may reside in its physical state as well as in certain chemical configurations.

#### Effects on Lymphoid-Reticuloendothelial System (RES) Tissue of Mice, Other Than Antibody Formation

Chart 2 summarizes the available information.

Studies are now in progress on the effects of MER on the lymphoid-RES tissue of other animals, on the total and differential peripheral blood counts of several species, and on the receptivity of lymphoid cells of mice to the mitogenic action of several nonspecific stimulators (phytohemagglutinin, pokeweed mitogen, and several others). Further studies are also being done on the kinetics of phagocytosis of inert and pathogenic materials by macrophages from MER-treated animals.

The observations to date suggest that the efficacy of MER does not depend on gross changes induced in lymphoid tissue at the site of eventual challenge, as appears to be so with some mycobacterial entities (16).

The findings that MER treatment stimulates macrophage phagocytic and enzymatic activity

CHART 2.—Effects of MER on lymphoid-RES tissue of mice, other than antibody formation

Enlarges and activates (notably, paracortical hyperplasia) draining, but not distal, lymphoid centers (4, 11).

Increases size, weight, and cellularity of spleens after intraperitoneal administration (12).

Rapidly increases RES clearance of colloidal carbon (both *k* and *a* values) and prepares animals for much later anamnestic clearance response after secondary stimulus with MER and other RES stimulators (12). Treated animals show markedly facilitated intraperitoneal clearance of bacterial pathogens (13).

Heightens rapidity of uptake, degradation, and elimination of labeled antigen (nonaggregated <sup>125</sup>I-BGG) from circulation, and alters early patterns of organ distribution (4, 14).

Passive transfer studies suggest effects on both lymphoid and macrophagic cells (15). Levels of macrophagic lysosomal enzymes (acid phosphatase, cathepsin D) increase 20- to 100-fold in treated animals, even after *in vitro* contact (4).

may be of direct significance to the modulating effects of the fraction on specific immunologic responsiveness: Processing by macrophages may be a necessary, or at least an ancillary, step in the "processing" of "raw" antigens toward full immunogenicity. Moreover, effective immunogenicity may be closely related to the ready access of an antigen to immunologically functional tissue, and also to a rapid reduction in the levels of unprocessed antigen to thresholds below those at which such material can evoke specific tolerance.

Thus, the data already accrued on the influence of MER on lymphoreticular tissues may illustrate stages in the sequence of events by which the fraction impinges on specific immunologic recognition and reactivity.

### Resistance to Microbial Pathogens

The ability of pretreatment with MER to elevate states of refractoriness to microbial infection is summarized in chart 3.

Further studies are in progress in several direc-

CHART 3.—Effects of pretreatment with MER on resistance to microbial pathogens

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Heightens resistance to experimental tuberculosis in mice and guinea pigs (including inhalation infection), at least to same extent as does living BCG, and acts synergistically in suboptimum amounts with quantities of methanol extracts not effective by themselves (6, 17).

Heightens resistance of mice to experimental infection with other, unrelated bacterial pathogens (*Pasteurella*, *Klebsiella*, *Salmonella*, *Staphylococcus*, *Pneumococcus*, *Proteus*, and *Pseudomonas*) (13, 18).

Heightens resistance of mice appreciably against endemic pneumotropic viruses under colony conditions (13).

Protection is frequently marked and manifested against large challenge inocula (13).

Protection is often manifested very soon after pretreatment and is maintained for many weeks or months (13).

Single pretreatment is often as or more effective than repeated administration (13).

Some protection is elicited sometimes even when treatment is initiated after infection (13).

In many systems, optimum protection effects are dose dependent; optimum dose is sometimes within narrow range; suboptimal and supraoptimal quantities do not, however, induce negative phases of resistance (13).

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tions: to ascertain the effects of MER on well-established bacterial infections; to extend information on the effects against viruses and on the production of interferon; and to study the conduct of the substance in protozoan host-parasite relationship systems.

From the information already available, MER seems to exert a broad protection, which is often dramatic, against microbial pathogens. It may indeed prove useful clinically in providing nonspecific protection, perhaps for limited periods, to individuals confronted with a high risk of serious infections because of new environmental circumstances or a fall in innate resistance. Thus, for example, MER may protect significantly against the side effects of therapeutic irradiation and cancer chemotherapy.

### Resistance to Neoplastic Cells in Mice: Pretreatment *Vis-à-Vis* Neoplastic Isografts

Many experiments have been conducted to define the pretreatment protective effects of MER, under various experimental conditions, against challenge with syngeneic neoplastic grafts in mice. The results of these experiments are summarized in chart 4.

The extensive findings condensed in chart 4 not only indicate the wide range of MER protective action against subsequent implants of syngeneic neoplastic tissue, but also point to the possibility that, under certain circumstances and against some tumors, MER pretreatment has the opposite consequence: facilitation of tumor growth. However, such unfavorable conditions can be corrected; thus joint specific immunization and nonspecific stimulation can abort the tendency to accelerated neoplastic development.

### Resistance to Neoplastic Cells: Effects on Spontaneous Tumor Development

As chart 5 shows, administration of MER can prevent the appearance of grossly detectable neoplasms in the autochthonous host exposed to carcinogenic stimuli, *if* the appropriate parameters of administration are found. However, changes in the dosage of MER and in the schedules of treatment can reduce or abrogate the protective action and, under some circumstances, again accelerate tumor development. These observations and the ones dis-

CHART 4.—Effects of pretreatment with MER on resistance to neoplastic isografts in mice

Markedly heightens resistance to various neoplastic isografts, including spontaneously arising and induced neoplasms, both in early and in late transplant generations (fibrosarcomas, hepatomas, mammary adenocarcinomas, osteogenic sarcomas, and uterine sarcomas) (19).

Resistance is often marked and expressed by one or more manifestations: retarded tumor growth; failure of isograft to take; regression after growth initiated; prevention or retardation of metastatic spread; and prolongation of life (19, 20).

Protective effects sometimes become evident shortly after treatment but usually several weeks must elapse for optimum effect. Protective effects are often of long duration (19).

Optimum effective dose is often narrow (19).

At supraoptimal doses and toward some tumors even with amounts maximally effective against most neoplasms, pretreatment accelerates tumor development, which appears, at least sometimes, to be classical immunologic enhancement (19, 21). Accelerated tumor development after pretreatment appears to be most likely in tumors which can induce enhancement by specific immunization (21, 22). However, animals treated with MER and specific tumor immunization do not show accelerated tumor development, but commonly show heightened tumor resistance; this is so even for tumors with an enhancing tendency (23).

Resistance is manifested against neoplasms even when pretreatment fails to trigger first- or second-set normal-tissue isograft rejection; *i.e.*, the effects are specifically directed at tumor-associated antigen(s) (19).

cussed in the preceding section thus focus on the balance of resistance-inducing and enhancement-inducing factors as perhaps the most significant equilibrium determinant of the fate of a newly arisen population of neoplastic cells (24–26). Any immunologic manipulation, specific or nonspecific, must thus always be considered in the light of the possibility that the equilibrium might be altered in the direction of enhancement-causing factors.

Such enhancement may be of a classic immunologic type, *i.e.*, the production or overproduction of antibodies capable of attaching to the neoplastic target cells but lacking the ability of destroying these cells, and thereby interfering with sensitization of the host or with the attack of more potent host immunologic factors, cellular or humoral. Immunologic enhancement in classical terms may also be expressed by an antibody-mediated central inhibition of further, or different, immuno-

logic reactivity, or by a paucity of antiblocking antibodies (35). In addition, other mechanisms of tumor facilitation, brought about by the insertion of a nonspecific immunologic modulator, must be considered. Thus, the modulator may, if in itself antigenic, as is the case for MER, pre-empt immunologic reactivity away from tumor-associated antigens. Alternatively, the local inflammatory reactions around foci of modulator deposits may act as “sinks” (3), nonspecifically draining away from availability in other areas, such as the tumor foci, immunocompetent elements. Other epi-immunologic mechanisms may be imagined.

Whatever may be the mechanisms of the tumor facilitation brought about occasionally and under rather defined circumstances by treatment with a nonspecific modulator, predictive means must be developed to pinpoint the adverse conditions. It may well be that, although some cancer patients in the limited trials so far reported appear to have benefited by treatment with nonspecific immunologic modulators, others may have been harmed by an acceleration of their neoplastic process. A shotgun approach to cancer immunotherapy raises obvious ethical and medical doubts. A question of

CHART 5.—Effects of treatment with MER on spontaneous tumor development

Treatment with low doses early in life reduces and delays development of palpable mammary carcinomas in multiparous C3H females. Large doses may enhance tumor development (27).

Markedly heightens resistance to development of preneoplastic hyperplastic alveolar nodules of mammary parenchyma of C3H mice under excessive hormonal stimulation (27). Stimulates immune response against premalignant mammary isografts (28).

Markedly heightens resistance to leukemogenic action of radiation leukemia virus in C57BL/6 mice, but only under certain parameters of administration; presence of some transformed cells at time of MER administration may be conditional on efficacy (4, 29). Protective activity in other viral leukemogenesis systems was reported by other workers (30).

Heightens resistance to development of skin papillomas in mouse skin isografts exposed to 3-methylcholanthrene and to carcinomatous transformation (31–33).

Sometimes heightens resistance of wild rabbits to papilloma and carcinoma formation induced by Shope papilloma virus (34).



major importance today is: Can reasonable predictive assessments of the directions of the effects of a nonspecific modulator of immunologic reactivity be made available as guidelines for the clinical investigator?

Much of our recent efforts have been in this direction, and the answer would appear to be, tentatively, affirmative based on the following considerations:

1) Certain consistent patterns of the consequences of MER treatment for resistance to neoplastic cells are emerging from extensive animal experimentation designed to create near models of human neoplasia. Thus, it seems that MER-treated mice *also* given specific tumor immunization, therapeutic irradiation, or tumor chemotherapy almost never show facilitated tumor development and frequently exhibit retardation of the neoplastic process (*see* next section). Although extrapolation from the laboratory animal to the human situation may be problematical, some framework to guide initial clinical attempts with nonspecific immunostimulators may be provided.

2) As described below, extensive studies in several species of laboratory animals have indicated certain parameters of MER treatment and specific immunization which strongly favor cellular over humoral immunologic responsiveness. Again, despite the inherent difficulties of extrapolation to man, these findings at least suggest initial guidelines.

3) Each cancer patient treated with MER or other such agents may perhaps be affected differently with regard to the resistance-enhancement equilibrium *vis-à-vis* a given population of tumor cells, and perhaps variably at different times during the disease. It is possible, however, to subject frequently each patient to a broad battery of test designed to establish his immunologic profile, and thereby to analyze the effects of MER on humoral and cellular reactivity against test antigens and against his own tumor cells. This process, although costly and time-consuming, places virtually no additional burden on the patient. As described below, we have developed such a battery, which appears to provide an ongoing reflection of each patient's differential immunologic status. Such individual monitoring may be significant even if some of the means of tumor acceleration induced by an extrinsic agent are not of the nature of classic immunologic enhancement: By directing specific

immune reactivity toward cellular elements capable of destroying neoplastic cells, the balance may be tilted in favor of the patient *even if* the modulator simultaneously initiates other phenomena in themselves inimical; the issue becomes one of overshadowing negative effects by positive ones.

Immunologic enhancement may be a basic regulatory mechanism for preventing the "horror auto-toxicus" of ongoing autoimmune reactions (36), and the pre-emption of this fundamental immune modality by cancer cells can well be imagined (22). Total prevention of elements of immunologic enhancement or their exclusion from the range of activities of nonspecific immunologic modulators may thus not be readily feasible. What may be possible, however, is the empiric definition of ranges of test circumstances which minimize enhancement phenomena in relation to the stimulation of resistance factors, and also the immunologic monitoring of individual patients so as to be able to adjust immunologic manipulation even more precisely to individual idiosyncrasy.

### **Resistance to Neoplastic Cells in Mice: Effects of Immunotherapy**

Many experiments have been conducted during the past year on the effects of MER in several strains of mice against the further growth of already established isografts of several different tumors, and against the development of simulated local recurrence and metastatic neoplasms. To mimic the clinical situations of local recurrence and metastasis experimentally, primary implanted tumors were removed surgically, and relatively small numbers of cells from that tumor reimplanted locally or injected intravenously. The formation of new growths at the site of removal of the first tumor was followed by periodic visual examination. When metastasis occurred, subgroups of mice were killed at different intervals, and their lungs were examined both by visual inspection and by quantitation of the uptake of labeled DNA precursors, which localize preferentially in rapidly growing tissues (37). The results obtained so far in these experiments, varying widely the conditions of treatment, are outlined in chart 6.

The findings summarized in chart 6 point to MER as an effective immunotherapeutic agent against cancer cells, especially when it is administered in conjunction with standard therapy. Indeed, a ma-

CHART 6.—Immunotherapeutic effects of MER in inbred mice\*

On occasion, MER by itself heightens resistance to the further growth of already established tumor isografts; enhancement is not induced, or only rarely. MER heightens resistance synergistically or additively with therapeutic irradiation and with chemotherapy and enables the animals to tolerate larger doses of radiation. Resistance is manifested by retarded tumor development and by prolonged survival.

MER alone more frequently and markedly prevents local recurrence and lung metastases; sometimes protection is the same or greater than that elicited by irradiation and chemotherapy. Combined treatment is frequently synergistic or additive. Local recurrence and metastatic tumors are entirely prevented in some animals, under some circumstances of treatment.

Small quantities of MER are more effective than larger ones. Intraperitoneal or subcutaneous administration distal to the implanted tumors is effective. Repeated treatment with MER alone and with MER plus irradiation or chemotherapy is sometimes effective even where a single treatment cycle fails to elevate resistance.

Treatment before surgical removal of primary tumor, or shortly thereafter, is generally more effective in retarding local recurrence than treatment delayed for some time after surgery.

\* Early observations on MER immunotherapy of tumors are reported in (19); most of the work summarized here will be reported in (4, 37-39).

for therapeutic contribution of MER might be the facilitation of more intensive radiation treatment and chemotherapy, by reducing the insults of such treatment to the immunologic apparatus of the patient. Standard cancer therapy must be viewed as a two-edged sword, and avoidance or marked reduction of the negative aspects may by itself considerably improve the possibilities of conservative treatment. It is hoped, in addition, that effective nonspecific immunotherapy, which is based on different mechanisms than those of irradiation and treatment with cytotoxic drugs, may eliminate clones of neoplastic cells resistant to radiation and drugs.

### Initial Studies in Cancer Patients of Effects of MER

The medical and ethical considerations which must be weighed in approaching clinical trials of an immunotherapeutic agent in patients with neoplastic disease have recently been detailed in (4).

Trials with MER in patients were initiated on the basis of the following considerations. The consistent observations in animals that MER does not induce enhancement when given jointly with standard treatment or with specific tumor immunization (it may be that the destruction of malignant cells effected by ionizing radiation and by cytotoxic agents results in the release of tumor-associated antigens and thereby facilitates hyperimmunization of the tumor-bearing subject) appeared to reduce this most prominent danger of immunologic intervention. Studies in guinea pigs (*see below*) also suggested that, when the specific antigen is weakly immunogenic, pretreatment with small quantities of MER directs the immune response preferentially toward cellular immunity. Lastly, it seemed possible to develop a broad immunologic profile test battery for monitoring each patient's immunologic status during treatment.

The limited clinical study undertaken so far has been confined largely to adult patients with acute myelogenous leukemia. MER treatment is given once a month by intradermal injection in 5-10 sites, of a total of 1 or 2 mg. Patients are randomly distributed into the MER-treatment and placebo-control groups, and all patients are maintained on standard therapy. Injections of MER are begun when the patient has been brought into remission by conventional treatment.

The battery of immunologic monitoring tests, performed every 2 weeks, consists of the following: total hematologic examination; qualitative and quantitative immunoelectrophoresis of serum proteins; quantitation of IgG and IgM titers to 2 test antigens, keyhole limpet hemocyanin (KLH) and a conjugate of dinitrophenol-human serum albumin (DNP-HSA), with which the patient is immunized after reaching remission; transformation of lymphocytes in the presence of phytohemagglutinin, a pool of normal human lymphocytes, and the patient's own leukemic cells obtained before remission; and testing of hypersensitivity skin reactions to a number of antigens, including purified protein derivative (PPD), a membrane preparation of the patient's own neoplastic cells, KLH and DNP-HSA, and a series of environmental allergens.

The number of patients under study for more than a few months is still too small to state the effects elicited by MER. There is the impression that some of the MER-treated patients remain in total remission considerably longer than the con-



trol patients and show an elevated immunologic reactivity, especially of IgM and cellular responses; it is too soon to say, however, whether this early impression will be borne out by further findings.

Injection of MER is well tolerated, the local reactions varying from mild and transient inflammation to a limited granuloma formation similar to a moderate living BCG reaction. Regional lymph nodes are on occasion slightly enlarged for a short period after MER administration, and several patients have had transient febrile episodes after repeated injection. No other adverse systemic effects have been noted, and MER clearly does not seem to cause any exacerbation of the leukemic process.

The studies are now being extended to patients with other forms of hematopoietic tissue neoplasia; if the first impressions of no negative effects and of some actual benefit are maintained, patients with solid tumors will eventually be included in the trials.

The emphasis in these human studies is to obtain a picture in depth of both the immunologic and the clinical consequences of MER treatment, rather than to collect merely clinical impressions on a large test population. The information to be gathered from this limited but intensive human investigation hopefully will provide more rational criteria for nonspecific immunotherapeutic treatment.

### Effects of MER on Immunologic Responsiveness to Known Antigens

Although the impact of MER treatment on host resistance to microbial and neoplastic invaders is most probably immunologic, other contributory mechanisms could not be discounted, and proof that the fraction does indeed have an immunologic locus of action had to be provided. Thus the effects elicited by MER on differential immunologic function to known antigens were examined under experimental conditions similar to those governing the involvement of MER in host-parasite relationships. Many such experiments have been conducted and are summarized in the following sections.

#### *Effects on Antigen-Reactive Cells*

Lymphoid cells which form rosettes with foreign red blood cells (RFC) can be separated experimentally into antibody-producing and non-antibody-producing varieties; the nonproducing variety may

CHART 7.—Effects of pretreatment with MER on the appearance of non-antibody-producing RFC in mice\*

Increases the numbers of both  $\theta$ †-positive and  $\theta$ -negative RFC, both in mice immunized with SRBC and in non-immunized animals (background RFC).

Increased RFC levels are maintained for at least several weeks.

MER stimulatory effect is most marked in immunized mice given small immunizing dose of SRBC.

Markedly protects against the depressive effects of treatment with cortisone acetate given after SRBC immunization on the appearance of RFC, especially of  $\theta$ -positive ones.

\* These observations are detailed in (4, 44).

†  $\theta$ : antigenic marker of mouse thymus and thymus-derived lymphoid cells.

be identical with the antigen-reactive cells (ARC) which possess specific surface receptors and whose reaction with antigen initiates the sequence of events leading to active antibody formation (40-43). The effects of MER on the appearance of non-antibody-producing RFC in mice immunized subsequently with sheep red blood cells (SRBC) are indicated in chart 7.

The experiments abstracted in chart 7 are still only in beginning stages, but the results already obtained indicate that MER may act during the very early as well as the later stages of the response to antigens, and it may indeed be that the later effects (described below) are in part the direct outcome of the activities brought to bear on ARC or on ARC precursors.

#### *Effects on Antibody Production*

Many experiments have been done in several strains of mice and other animals to ascertain the effects of MER treatment on the levels of specific plaque-forming cells (PFC) and on free antibodies to a number of different antigens. The most important findings are summarized in chart 8.

Over a broad range of experimental parameters, MER stimulated, sometimes markedly, circulating antibody production in mice. However, the manifestations of the MER effect on the formation of antibodies took on different forms under different circumstances. Thus, for instance, secondary responsiveness to SRBC was frequently delayed, though ultimately stimulated, whereas secondary



CHART 8.—Effects of treatment with MER on the production of antibodies to known antigens

Pretreatment accelerates the onset, prolongs the duration, and increases the levels of the primary response, both IgG and IgM, to subsequent immunization with foreign RBC, KLH, and T2 phage in mice (12, 45, 46). Stimulation is most marked when specific immunization is with limiting quantity of antigen (47).

Heightens an already ongoing primary response in mice to SRBC (47) when immunization is with limited numbers of the red blood cells.

Restricts in mice IgG feedback inhibition of IgM response (4, 48).

Increases levels and duration of the secondary response in mice, both IgG and IgM, to SRBC, KLH, and T2 phage, but often delays onset of the secondary response to SRBC. These effects are elicited when MER treatment precedes primary immunization and also when given between primary and secondary antigen contact (45–47).

Pretreatment stimulates formation of antibodies in guinea pigs to DNP-protein conjugates not by themselves strongly immunogenic, but only under certain well-defined experimental conditions (under other circumstances MER pretreatment favors development of delayed hypersensitivity) (49).

responsiveness to other test antigens was facilitated without an intervening lag period.

With the one exception of young, outbred, albino mice treated with MER very shortly before SRBC immunization (4, 12), MER treatment never reduced antibody reactivity in this species. Even administration at the time of specific immunization and sometimes thereafter had a stimulatory effect. [In guinea pigs, (*see also below*), however, MER pretreatment could selectively favor humoral antibody formation or delayed hypersensitivity to hapten-protein conjugates, depending on the precise conditions of treatment and specific sensitization; it may well be that in other species as well, and for other antigens, such preferential stimulatory activity by MER will become evident.]

The delay in secondary responsiveness of mice *vis-à-vis* the test antigen SRBC was specific: The same animals showed simultaneously an unimpeded responsiveness, both primary and secondary, to another test antigen, T2 phage (4, 45, 48). The initial inhibition of the secondary response to some (large, particulate?) antigens, the hastening of peak first responses to all antigens tested, and the restriction of IgG feedback inhibition of IgM synthe-

sis all point to an exaggeration of the IgM component of the antibody reaction, at different stages in the ontogeny of a given response. Many investigators have ascribed particular resistance value to antibodies of the IgM class, which are strikingly more effective in complement-fixing and cytotoxic activities; the prominence given to the expression of this type of immunoglobulin by MER treatment may well be one of the fraction's modes of action.

Analysis of the effects of MER on numbers of specific PFC in lymphoid tissue and on titers of free antibodies suggests that MER acts at different loci in the process of antibody formation: The numbers of specifically reactive lymphoid cells may be increased and their antibody-synthesizing capacity enlarged; furthermore, the liberation of formed antibody molecules may be facilitated. Alternatively, or in addition, the activities of MER may be focused, directly or indirectly, at the stage of antigen processing by macrophagic cells, and studies are in progress to define the cell type on which MER exerts its initial activities. The findings to date indicate that both lymphoid cells and macrophages are affected early, but a truly primary effect on one or another of these families of cells has not yet been established in any system.

The modes of action of MER on humoral antibody production are discussed further in other recent communications (45–47, 50).

Preliminary observations also suggest that MER facilitates the production of antibodies in other species (4).

### *Effects on Cellular Immunity*

Because the rejection of foreign tissues, including antigenically variant, autochthonous, neoplastic cells, is generally thought to depend primarily on cellular immune reactions,<sup>6</sup> much effort has re-

<sup>6</sup> Care must be taken, however, not to oversimplify the issue of the relative importance of cellular and humoral elements in homograft reactivity and to remain receptive to newer information. Thus, for example, it has recently been proposed that specifically sensitized lymphoid cells may in some test systems enhance tumor development (51). Conversely, there has long been the impression that humoral antibodies may have at least an auxilliary role in the attack on foreign solid tissues and perhaps a primary role in the destruction of dispersed, alien cells; recently, certain subclasses of IgG antibodies have again been incriminated in solid tissue rejection (52). The weight which should correctly be given to cellular and humoral immune elements in tumor resistance thus is not entirely clear.

cently been given to analyze the effects of MER on cellular immunologic responsiveness.

In preliminary studies on the effects of MER treatment on cellular immunity, experiments were performed in the system of male skin isografts to female mice of several different strains. Pretreatment with MER increased the frequency of transplant rejections and reduced the survival time of rejected grafts (53).

Two other models were subsequently investigated intensively because they are easier to quantitate: In the first model, mice of several inbred strains were immunized with an allogeneic plasma cell tumor (PCT) after treatment with MER or placebo, and the cytotoxic capacity of their lymphoid cells was subsequently assayed against the PCT targets *in vitro*; the lymphoid cell killer activity was ascertained by following the release of  $^{51}\text{Cr}$ , with which the target cells were labeled (54). In the other model, guinea pigs were sensitized with DNP conjugates of homologous serum globulin (GPG) or heterologous serum albumin (HSA), with and without MER treatment, and their ability to develop delayed hypersensitivity and/or humoral antibodies to the antigens was ascertained. DNP-GPG and DNP-HSA are poor immunogens in the guinea pig, and single sensitization did not lead to detectable responses unless the antigen was administered in complete Freund's adjuvant (CFA). Delayed hypersensitivity was ascertained by skin tests with the antigens, and humoral antibody formation by an indirect hemagglutination test.

The results obtained in the PCT allograft model are summarized in chart 9.

Under various test conditions, pretreatment of C57BL and C3H mice heightened the cytotoxic

efficacy of their lymphoid cells (from spleen, lymph nodes, and peritoneal washings) against a BALB/c PCT. Treatment as long as 1 month before PCT immunization was successful, as was treatment administered only several days preceding; both single and repeated administration of MER proved effective. Heightened cytotoxic efficacy was expressed by a greater liberation of target cell  $^{51}\text{Cr}$  within a set interval, by a shorter period of time required to obtain the release of a fixed amount of chromium, and by a lower lymphoid cell-target cell ratio required to free a given proportion of the label.

The effects seen in this system appear to accrue from direct cell-cell interactions, rather than from the liberation of soluble cytotoxic mediators (55).

More sensitive techniques have recently been developed in our laboratories for following the fate of target cells under attack by specifically immune lymphocytes *in vitro* (57). These are based on sequential determination of the uptake of labeled precursors of protein, RNA, and DNA synthesis by the target cells during the first several hours of contact. This uptake is inhibited very rapidly after attack, and changes in the slopes of precursor incorporation apparently can provide a sensitive indication of the early consequences of cytotoxic action by the lymphoid cells (57). Preliminary observations suggest that pretreatment with MER of the lymphoid cell donors also heightens the cytotoxic capability of the cells as indicated by these metabolic criteria.

In passive transfer studies, lymphoid tissue cells were taken from MER-treated donors which were not immunized with the PCT, and were given to irradiated, syngeneic recipients which were then sensitized with the allogeneic tumor cells. The lymphocytes obtained 10 days later from the recipients showed elevated anti-PCT activity. In a series of experiments in which purified populations of lymphoid and macrophagic cells were derived from MER-treated and normal donors and transferred passively to irradiated recipients subsequently immunized, either cell type from the MER-stimulated mice appeared to convey the effect. These observations show that terminally, at least, the effect of MER stimulation is manifest in lymphoid and macrophagic cells; this is borne out also by the observations (*see below*) that MER can reverse the immunosuppressive effects of antilymphocytic serum (ALS) in this system.

CHART 9.—Effect of pretreatment with MER on the cytotoxic capacity of mouse lymphoid cells against allogeneic plasmacytoma target cells *in vitro*\*

Consistently and appreciably increases the cytotoxic efficacy of lymphoid cells from specifically immunized donors over wide range of experimental circumstances; in preliminary experiments, also increased cytotoxic efficacy of lymphoid cells from normal donors.

Heightened cytotoxic efficacy can be transferred passively by both lymphoid and macrophagic cells from MER-treated donors.

\* Detailed in (55, 56).

TABLE 1.—Effect of treatment with MER on response of guinea pigs to DNP conjugates of GPG and HSA\*

Pretreatment	Immunization (in all 4 footpads, once)	DNP-GPG		DNP-HSA	
		DH	AB	DH	AB
None	In saline, 10 $\mu$ g	0	0	0	0
None	In IFA, 10 $\mu$ g	0	0		
None	In CFA, 1 $\mu$ g	+	0	+	+
None	In CFA, 10 $\mu$ g	+	+	+	+
None	In saline + MER, 1 $\mu$ g			0	0
None	In saline + MER, 10 $\mu$ g	0	0	0	0
None	In IFA + MER, 10 $\mu$ g	+	+		
CFA, in footpads, day -7	In saline, 10 $\mu$ g	+	0		
CFA, in footpads, day -14	In saline, 10 $\mu$ g	0	0		
CFA, ip, day -7	In saline, 10 $\mu$ g	0	0		
MER in IFA, ip, or footpads, days -7 or -14	In saline, 10 $\mu$ g	0	0		
MER in saline, ip, sc, id, or footpads, days -7 or -14	In saline, 10 $\mu$ g	+	±	0	+
MER in saline, ip, sc, id, or footpads, days -7 or -14	In saline, 1 $\mu$ g			+	0

\* Detailed in (49, 56). DNP-GPG = conjugate of dinitrophenol-guinea pig globulin; DNP-HSA = conjugate of dinitrophenol-human serum albumin; DH = delayed hypersensitivity, as indicated by typical skin test appearance; AB = antibody formation, as measured by passive hemagglutination test; CFA = complete Freund's adjuvant, containing killed mycobacteria; IFA = incomplete Freund's adjuvant; and ip, sc, id = intraperitoneal, subcutaneous, and intradermal, respectively.

The findings obtained so far in the guinea pig experiments are summarized in table 1. These studies include several hundred guinea pigs; all individual experiments were repeated, always with groups of at least 5 animals each. The interpretations of these findings are indicated in chart 10.

The information in table 1 and chart 10 clearly shows that MER possesses modulator qualities which go beyond a classical Freund's adjuvant effect, and that, depending on the parameters of the test system, either DH or antibody formation is the preferred response of treated guinea pigs to subsequent sensitization. The quantities of MER used in these studies were only 1 or 2 mg, and, indeed, exploratory experiments with 4 or 5 mg indicated a much reduced effect.

The heterologous protein-DNP conjugate is a somewhat stronger immunogen than the homologous one, as indicated from experiments in which sensitization is by repeated injection. Thus apparently *vis-à-vis* appreciable quantities of a very weak immunogen (10  $\mu$ g DNP-GPG) and *vis-à-vis* very small quantities of a somewhat stronger one (1  $\mu$ g DNP-HSA), pretreatment with small amounts of MER favors the development of cellular immunity (DH, in this model). It is not yet known to what extent these observations can be extrapolated to other systems. If the findings are more generally applicable, they may be of special interest with regard to the tumor-protective effects of MER: Tumor-

associated antigens are generally considered to be rather weak immunogens!

### Correction of States of Immunologic Deficiency by MER

In mice, MER can raise levels of immunologic capacity low because of age or the influence of specific and nonspecific environmental factors. The corrective and restorative activities of MER are broad and often marked. These effects are detailed in a recent review (4) on the behavior of the fraction, and are recounted briefly in chart 11. If the findings in mice can be confirmed in current studies with other animals, MER will emerge as an agent capable of modulating the immunologic responsiveness not only of immunologically normal subjects but also of immunologically debilitated animals.

### CONCLUDING COMMENTS

Even from this brief description, it is seen that MER influences profoundly both B-cell and T-cell function and both the humoral and cellular components of the immune response to a variety of soluble, particulate, and cell-bound antigens. Further work in mice and in other animals possibly will reveal distinct patterns of parameters of MER



CHART 10.—Behavior of MER in the guinea pig (DNP-protein conjugate system): Summary of recent experiments

Neither the homologous conjugate (DNP-GPG) nor the heterologous conjugate (DNP-HSA) is sufficiently immunogenic to evoke a detectable immune response by itself after single administration.

Administration of DNP-GPG in IFA also failed to evoke an immune response.

Administration of 10  $\mu$ g of both conjugates in CFA evoked both DH and antibody formation; both reactions were also manifested against 1  $\mu$ g of DNP-HSA, but only DH was elicited by the smaller amount of DNP-GPG.

Incorporation of MER in the saline-antigen mixture at the time of sensitization failed to evoke responsiveness, but substitution of MER for whole, killed mycobacteria in Freund's adjuvant elicited both responses (with DNP-GPG; the other conjugate not tested in this combination). MER can thus substitute for intact mycobacteria in a classical adjuvant situation.

Pretreatment with CFA did not enable animals to react to DNP-GPG, unless administration was at the site of subsequent sensitization and shortly preceding (7 days), in which case DH was induced.

In contrast, pretreatment with MER in a saline vehicle, by any of several routes, and whether 7 or 14 days before sensitization, conferred reactivity: When sensitization was with 10  $\mu$ g of the conjugate, strong DH but only very limited antibody responses were manifested against DNP-GPG, and good antibody responses but no DH against DNP-HSA. The reverse results were obtained when only 1  $\mu$ g of DNP-HSA was used: strong DH and no antibody reactivity. MER thus can prepare guinea pigs to respond to poorly immunogenic substances, in one or another direction, under conditions where CFA is wholly ineffective; MER clearly possesses modulator activity which goes beyond the classical Freund's adjuvant effect.

When MER was given as pretreatment, but incorporated in IFA, it exerted no preparatory effect. MER must thus be given in a "free" form to act as an effective pretreatment modulator.

treatment and specific immunization which are predictably determinant of the agent's modulation of responsiveness in different directions. It may also turn out, however, that the modulating activities of MER are so labile and fluid that the probable outcome of its effects under natural conditions shall have to be ascertained and followed empirically for a given subject. Although this last possibility would pose large technical difficulties to the intervention by MER in human disease, the problems do not seem insurmountable.

CHART 11.—Correction of states of immunologic deficiency in mice by MER treatment\*

Heightens inherently low immunologic capacity of very young and old mice to several test antigens (4).

Prevents induction of specific immunologic tolerance by unaggregated protein antigen (BGG) and facilitates active immunologic reactivity against such otherwise tolerigenic material (4, 50, 58).

Prevents partial secondary tolerance to SRBC after primary immunization with large numbers of RFC (4).

Protects at least partially against reduction in numbers of antigen-reactive cells brought about by treatment with anti- $\theta$  serum and cortisone (4, 44).

Reduces the immunosuppressive effects of X irradiation (in at least some systems, only when given before irradiation) (4, 48).

Reverses largely or completely the immunosuppressive effect of ATS on circulating antibody response to SRBC, whether given before or after ATS (4, 48), and, similarly, the immunosuppressive effect of ALS on the cytotoxic activity of lymphocytes against allogeneic tumor target cells (55).

\* BGG = bovine gamma-globulin; ATS = antithymocytic serum; and ALS = antilymphocytic serum.

The changes in specific immunologic reactivity and in states of resistance effected by MER are manifested under similar experimental conditions. Although the participation of nonimmunologic mechanisms nonetheless cannot be excluded rigorously, most probably the impact which MER exerts on the altered ability to withstand microbial and neoplastic challenge is causally related to its effects on immunologic function. It is also noted that MER much more commonly heightens opposition to neoplastic development than affects it adversely (*e.g.*, enhancement), that MER almost always elevates the ability to repel microbial parasites and never induces negative phases of resistance, and that the effects of MER on immunologic reactivity are nearly always reflected in the enlargement of one or another facet of the response. Unlike many other agents which act nonspecifically on resistance and on the immunologic apparatus, MER thus seems to be predominantly a "positive" stimulant. It clearly shares properties with other nonspecifically active substances, and especially with other mycobacterial entities, but there are aspects of its behavior which would seem to be categorically divergent, or for which analogous

conduct by other such agents has not yet been discovered.

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# Immune Suppression and Carcinogenesis in Hamsters During Topical Application of 7,12-Dimethylbenz[a]anthracene<sup>1, 2, 3</sup>

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**SUMMARY**—Regional and systemic immune responses were correlated with tumor development in Syrian hamsters treated with 7,12-dimethylbenz[a]anthracene (DMBA), a potent carcinogen. The results indicated a significant transient depression of regional and systemic humoral immunity, followed by a more lasting suppression of cell-mediated immunity. Papilloma development was associated with maximal suppression of humoral immunity, whereas malignant transformation began at the transitional period between recovery of humoral immunity and depression of cell-mediated immunity. The functional role of depressed humoral and cell-mediated immune responses in tumorigenesis is supported by results of studies on DMBA-induced *in situ* tumor development in heterologous antithymocyte serum-treated, splenectomized, and/or lymphadenectomized animals. Although initial papillomagenesis was primarily contingent on the early depression of humoral immunity, survival of papillomas in the last phases of their development appeared to be regulated through cell-mediated immune reactivity elicited directly in proportion to immunogenicity. These results showed that a specific chronologic relationship between the transient depression of regional and systemic humoral immunity and the more lasting depression of cell-mediated immunity is necessary for successful tumorigenesis. They also supported the contention that a *modulation* of normal humoral (regional and systemic) and cell-mediated immunity is induced by DMBA and is functional in tumorigenesis.—Natl Cancer Inst Monogr 35: 173–182, 1972.

A VARIETY of carcinogenic hydrocarbons, unlike their noncarcinogenic analogues, can depress hu-

moral and cell-mediated immune reactivity (1–4). The degree of immunosuppression (and tumor incidence) greatly depends on carcinogen dose and genetic susceptibility of the host (5–8). In general, the duration of carcinogen-mediated humoral immunosuppression corresponds to the period of tumor latency, whereas the depression of cell-mediated immunity is variable, as reflected by the different survival times of tumor and skin grafts having different antigenic characteristics (4, 9–15). The immunosuppressive effects of chemical carcinogens have generally been studied systemically; there is little information relating the immunologic

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status of draining lymph nodes to tumor development.

One of the central questions concerning the successful development of antigenic tumors is the degree and type of immunologic response available at the site of a given autochthonous malignant lesion. Due to the anatomic location of the developing tumor or to pathologic changes in the normal anatomy of associated tissues, tumor-specific humoral and cellular defense mechanisms are not always optimally available at the tumor site (16). By the examination of the status of systemic and regional immune responses during prolonged treatment with a chemical carcinogen, it should be possible to correlate a suppression of cell-mediated or humoral immunity (or both) with the different stages of tumorigenesis.

Using the carcinogen 7,12-dimethylbenz[*a*]anthracene (DMBA) and Syrian hamsters, we began systematic studies of *a*) the depression of systemic and regional humoral and cell-mediated immunity by DMBA as it relates to tumor development and *b*) the incidence and latency of tumors in immunologically competent and immunologically depressed animals during and after prolonged topical DMBA treatment of the skin.

## MATERIALS AND METHODS

**Animals.**—Male Syrian Cr:RGH hamsters (Sprague-Dawley, Madison, Wisc.), 4–5 weeks old, were used for the carcinogenesis studies. Male Chinese hamsters (Children's Cancer Research Foundation, Boston, Mass.), 6–8 weeks old, were used as donors for skin allografts. They were maintained 5 to a cage and were given free access to food and water.

**DMBA treatment.**—Animals were treated with DMBA (Sigma Chemical Co., St. Louis, Mo.) on an approximately 2-cm<sup>2</sup> area of the skin posterior to the right shoulder after the hair had been clipped. The chronic DMBA treatment consisted of topical application (with an automatic pipette) of 250 µg of DMBA in 25 µl of acetone twice weekly for 86 days. Treatment with acetone alone did not produce tumors or any detectable changes in the skin. The total number of treatments was 25, unless otherwise indicated.

**Histology.**—After each DMBA treatment, 2 or 3 animals were killed, and portions of their treated skin, as well as their draining and mesenteric

lymph nodes, spleen, thymus, bone marrow, liver, and adrenal glands, were prepared for histologic study.

The histopathology of DMBA-induced skin lesions and lymphoid organs was correlated with changes in the gross morphology of developing tumors. The criterion for malignancy was interruption of the epidermal basement membrane and invasion of the *panniculus carnosus*.

**Tumor latency and incidence.**—After each DMBA treatment, animals were examined for papilloma development, papilloma regression, and development of defined carcinomas. The numbers of papillomas and carcinomas were recorded weekly after the last treatment with DMBA. As soon as developing carcinomas were recognizable, biopsies were performed and the biopsy specimens were examined for invasiveness to confirm the character of the carcinomas histologically. The procedure of Lappé was used for diagnosis of gross malignancy (17).

**Immunization.**—The test antigen, sheep red blood cells (SRBC), was obtained sterile in Alsever's solution (Baltimore Biological Co., Baltimore, Md.), washed 3 times in 10–20 volumes of isotonic saline at 4°C, and resuspended in saline to give a 0.5 % suspension of erythrocytes. The injection of antigen was either intraperitoneal or intradermal, as indicated in the experimental plan.

**Serum hemagglutinin and plaque-forming cell (PFC) determinations.**—Hemagglutinin titers in the serum (for 19S and 7S antibody activity) and the plaque-forming response of 19S and 7S antibody-producing cells to SRBC in the spleen and in draining lymph nodes were determined 5 and 8 days after the injection of antigen, according to previously described procedures (18). Rabbit anti-hamster γ-globulin (Hyland Laboratories, Los Angeles, Calif.) was used to develop indirect plaques. The plaque counts were corrected for a 50 % inhibition of 19S plaques by the antiserum (18).

**Skin transplants.**—Depending on the experiment, a 6 × 12-mm area of full-thickness skin was transplanted to the right (ipsilateral) or the left (contralateral) side of the animal just posterior to the shoulder. Grafting and postoperative dressing were done according to techniques described by Billingham (19). Plaster casts were removed from the animal 7 days after grafting, and the status of the graft was assessed twice weekly. Scab formation following ulceration of the graft was taken as the



endpoint of allograft rejection. The results were expressed in mean survival time (MST).

*Antithymocyte serum.*—Heterologous rabbit anti-thymocyte serum (ATS) was prepared against hamster thymocytes according to the method of Levy and Medawar (20). After the antiserum was de complemented by being heated at 56°C for 30 minutes, it was absorbed twice on washed hamster erythrocytes. The proportion of packed red cells to serum was 1:2 (v/v). After adsorption, the serum was titrated against washed hamster thymocytes, nucleated spleen and bone marrow cells, and erythrocytes. The ATS titer against thymocytes and lymphoid cells of the spleen was  $\log^{-1}_2 9$ ; against bone marrow and red cells it was  $\log^{-1}_2 3$ . ATS was always injected intraperitoneally.

*Splenectomy and lymphadectomy.*—Spleens and/or lymph nodes were removed 1 month before treatment with DMBA. Spleens were removed through a 2-cm incision in the left abdominal wall. After the splenic artery and vein were cauterized, the incision was closed with autoclips.

The right distal axillary and the consecutive proximal axillary lymph nodes were dissected through a 2-cm incision near the axillary fossa to prevent draining of the site of subsequent DMBA treatment. After removal of the lymph nodes, the incision was closed with autoclips.

In sham-operated animals, a 2-cm incision was made on the right abdominal side and the underlying fat pad was manipulated with a blunt probe before the incision was closed with autoclips.

## RESULTS

### DMBA-Induced Histologic Changes in Lymphoid and Hematopoietic Organs

Histologic examination of tissues obtained from DMBA-treated hamsters revealed changes in the normal morphology of lymphoid and hematopoietic organs, consistent with the expected depressions in humoral and cell-mediated immunity. The number of lymphoid cells was reduced and germinal centers were absent in the non-thymus-dependent areas of the ipsilateral lymph node by the fifth DMBA treatment and in the splenic white pulp after the thirteenth treatment. Early in DMBA treatment, sinusoids were congested and the number of hematopoietic cells in the bone marrow was somewhat reduced. By the nineteenth treatment,

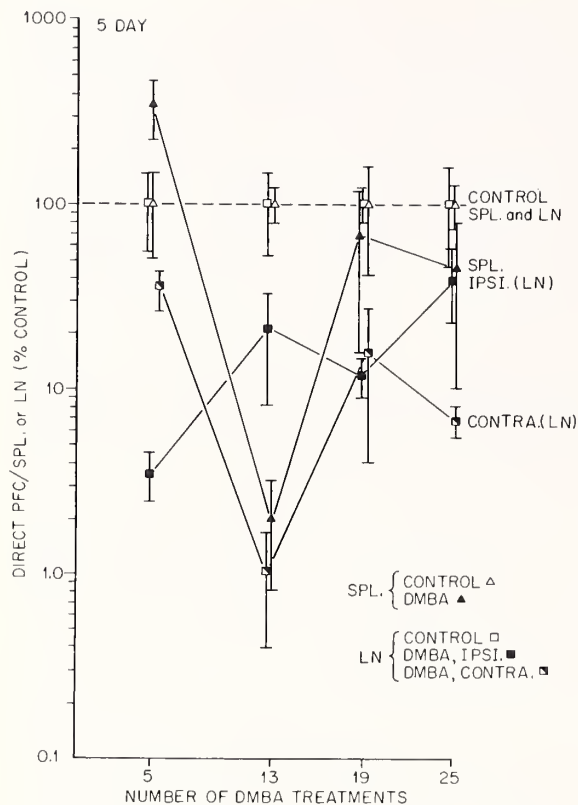
the number of cells in these organs returned to control levels and some germinal centers appeared in the spleen. Thymocytes were reduced in number, but only in the late stages of DMBA treatment. Several weeks after completion of DMBA treatment, the thymus and splenic white pulp of animals with advanced stages of squamous cell carcinoma were reduced in size and the ipsilateral lymph node contained occasional foci of squamous cell metastasis.

### Systemic and Regional Immunity During Chronic DMBA Treatment

#### *Humoral Immune Response to SRBC*

One hundred male Syrian hamsters, 4–5 weeks old, were used to determine the effect of DMBA on humoral immunity. Animals were given injections of SRBC in groups of 5, one day after 5, 13, 19, or 25 DMBA treatments. Although a thymus-dependent antigen, SRBC was not expected to reflect changes in the thymus-dependent component of the humoral immune response. This contention was based on preliminary data showing the lack of sufficient histologic changes in the thymus and thymus-dependent areas of spleen and lymph nodes and the lack of any depression of cell-mediated immunity, until the nineteenth and twenty-fifth DMBA treatments. For determination of systemic immune response, 1 group of animals received 1 ml of 0.5% SRBC intraperitoneally. For determination of regional immunity, 2 additional groups of animals were given intradermal injections of 0.5 ml of 0.5% SRBC on the ipsilateral or contralateral side. Control groups of untreated animals received similar doses of SRBC, either intraperitoneally or intradermally. Whether expressed per organ or  $10^6$  nucleated cells, the observed changes in number of PFC's did not differ significantly. The average number of PFC's per spleen or lymph node, with standard error, was plotted as percent of control (text-fig. 1).

A reduction in the number of direct PFC's (from a control level of 3,800 to 130) was first observed after the fifth DMBA treatment in the draining ipsilateral lymph node, as assayed 5 days after the injection of antigen. The number of PFC's returned to control levels by the thirteenth treatment. A significant depression in the 5-day direct PFC's was also observed for the contralateral lymph node



TEXT-FIGURE 1.—Comparison of PFC activity in DMBA-treated and control lymphoid organs 5 days after injection of the test antigen (SRBC). SPL., spleen; LN, lymph node; IPSI., ipsilateral (DMBA-treated side); and CONTRA., contralateral.

and spleen after the thirteenth treatment (text-fig. 1). It involved a drop in the number of PFC's from a control level of 8,600 to 89 for the contralateral draining node and a reduction from 60,000 to 1,200 for the spleen. After the nineteenth and twenty-fifth DMBA treatments, the average number of PFC's in the spleen and lymph nodes did not differ significantly from control values.

The effect of DMBA treatment did not produce a consistent, significant depression of the 7S response to SRBC. However, the indirect plaque-forming assay in hamsters was less than adequate in our hands. The limitation of the technique could possibly be attributed to an ineffective facilitating serum preparation. Therefore, we do not feel justified in interpreting the 7S response.

Thus, after chronic DMBA treatment, the 19S humoral immune response was significantly affected. A transient 97% depression of the 19S response occurred first in the regional lymph node

draining the site of DMBA treatment. A similar, 98% depression of the 19S response was observed later (44 days after the first treatment) in the more distant contralateral node and spleen. The sequence and extent of these depressions are important in understanding the dynamics of the DMBA-modified systemic versus regional immunity. Hemagglutinin titers confirmed the above observations.

#### *Cell-Mediated Immune Response to Chinese Hamster Skin Allografts*

For study of the effect of DMBA on cell-mediated immunity, 60 male Syrian hamsters, 4–5 weeks old, were divided into 3 equal groups. One group of DMBA-treated animals received male Chinese-hamster skin allografts ipsilaterally; the other received them contralaterally. The third group received skin grafts but no DMBA. Grafting was always done on animals in groups of five 1 week after 5, 13, 19, or 25 DMBA treatments or at equivalent ages for the untreated controls. Isografts were used as technical controls. After grafting, the results were plotted as MST  $\pm$  SE (text-fig. 2) and then were correlated with the humoral immune status and tumor development in DMBA-treated animals.

Male Chinese hamster skin transplanted to normal male Syrian hamsters was consistently rejected after survival of 8–13 days after grafting, depending on the age of the recipient. The MST of allografts on DMBA-treated Syrian recipients increased with continued DMBA treatment; the first significant increase (to  $42 \pm 10$  days) was observed after 19 DMBA treatments. After the last, or twenty-fifth treatment, the MST was  $60 \pm 9$  days (text-fig. 2). The MST did not differ significantly between ipsilateral and contralateral grafts.

Since allograft rejection is predominantly the result of cell-mediated immunity, a relative percentage for the depression of cell-mediated immunity during chronic DMBA treatment was calculated. This was done by subtracting, from 100%, the value of control allograft MST, expressed as percentage of MST of allografts on (equivalent age) DMBA-treated recipients. This relative percentage was approximately 72% after 19 DMBA treatments and 86% after 25 treatments; thus the degree of depression of cell-mediated immunity in these experiments was directly proportional to the increase in the number of DMBA treatments.

### Correlation of Tumor Incidence and Immunosuppression

Depression of regional humoral immunity in the ipsilateral distal axillary lymph node, evident after the fifth DMBA treatment, preceded the appearance of the first skin lesions. Papillomas and melanotic lesions became grossly detectable by the thirteenth DMBA treatment, when depression of humoral immunity in the spleen was maximal. During the recovery phase of systemic humoral immunity (between the thirteenth and nineteenth DMBA treatments), the incidence of papillomas and melanotic lesions increased exponentially. By the nineteenth DMBA treatment, when the depression of cellular immunity first became significant, some papillomas had progressed to the carcinoma stage. Although directly correlated with carcinoma development, the suppression of cellular immunity in our experiments must be attributed to DMBA treatment, since there is a low incidence of tumors at a time when all animals have suppressed cell-

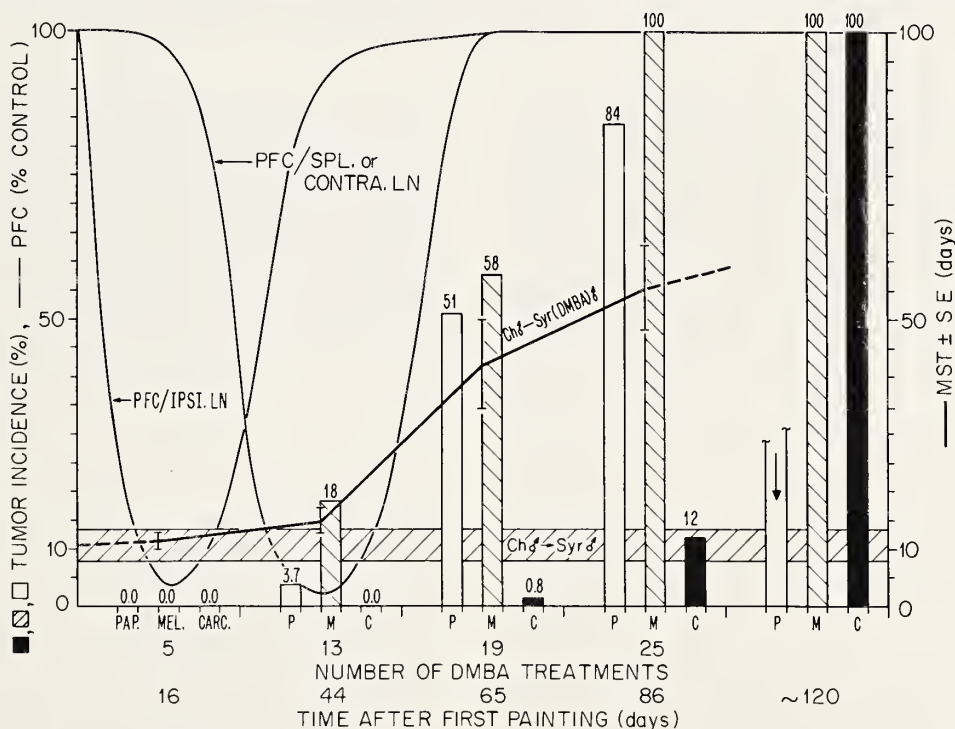
mediated immunity. Further carcinoma development paralleled the depression of cell-mediated immunity. Malignant transformation of melanotic lesions was not observed.

### Tumor Incidence and Latency in Immunologically Suppressed Animals During Chronic DMBA Treatment

If depression of humoral or cell-mediated immunity is functional in DMBA carcinogenesis, then suppression of immunity before DMBA treatment should enhance tumor development. To test this assumption, we treated groups of animals with 2 different regimens of ATS or removed their spleens and/or lymph nodes.

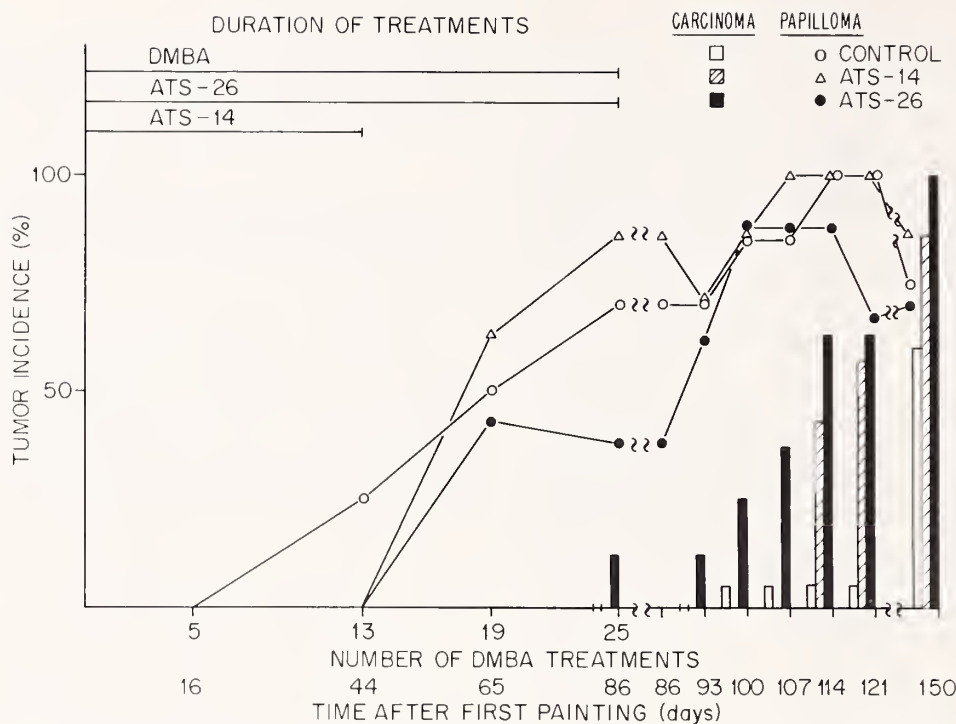
#### Effects of ATS on Tumor Development

Sixteen animals were given intraperitoneal injections of 0.5 ml of ATS 4 days before DMBA



TEXT-FIGURE 2.—Correlation of the sequential depression of regional and systemic humoral immunity with allograft mean survival times (MST  $\pm$  SE) and the latency and incidence of papillomas, melanotic lesions, and carcinomas in DMBA-treated hamsters. IPSI., ipsilateral (DMBA-treated side); CONTRA., contralateral (untreated side); SPL., spleen; LN, lymph node; Ch, Chinese hamster donor; Syr, Syrian hamster recipient; P(PAP.), papilloma; M(MEL.), melanotic lesion; and C(CARC.), carcinoma.





TEXT-FIGURE 3.—Papilloma and carcinoma incidences during and after DMBA treatment of animals given injections of rabbit anti-hamster thymocyte serum (ATS) or given DMBA alone (controls).

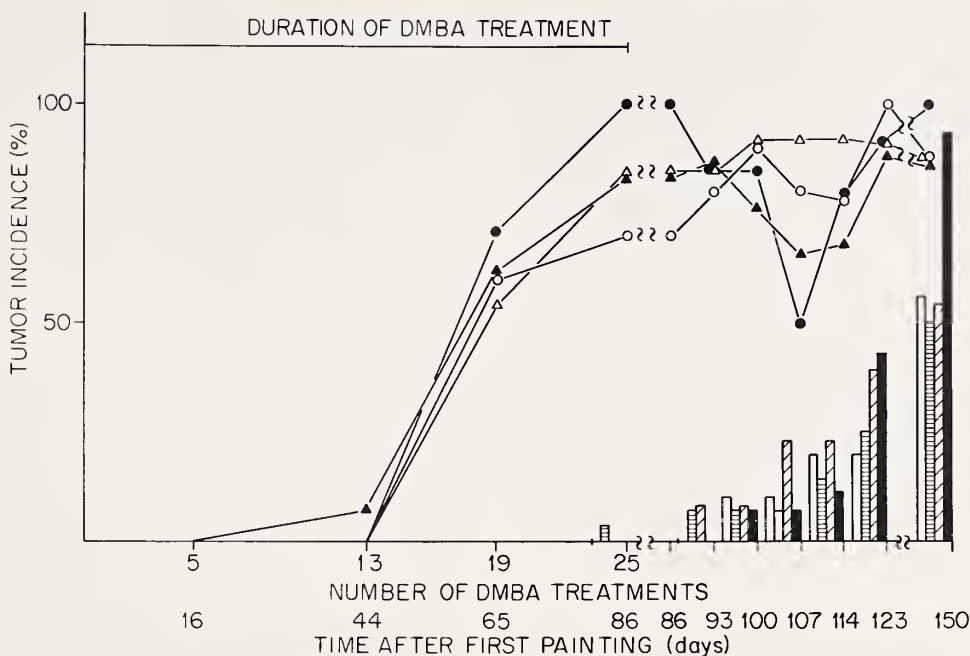
treatment was begun; they received 6 more injections simultaneously with DMBA application. After a total of seven 0.5-ml injections, the animals received additional 0.25-ml intraperitoneal injections of antiserum through the thirteenth DMBA treatment, when papillomas first appeared in the control group (30 animals treated with DMBA only). At this time, ATS treatments were discontinued for half the animals but were continued for the remainder through the twenty-fifth treatment of DMBA, in parallel with papilloma development. The group with a total of 14 ATS injections was designated ATS-14, while the one with 26 injections was designated ATS-26. Text-figure 3 illustrates the effects of the 2 different regimens of ATS on papilloma and carcinoma development.

When ATS was administered during papilloma development, a significant papilloma regression (55%) was observed in the ATS-26 group between the nineteenth and the twenty-fifth DMBA treatments. These regressions reduced the overall incidence of papillomas, since in the animals treated continuously with ATS (ATS-26) more papillomas were regressing than appearing (text-fig. 3). In contrast, the incidence of papillomas in the ATS-14

group increased to 86% by the twenty-fifth DMBA treatment (text-fig. 3). Papilloma regressions during this period in the control group and in the ATS-14 group were insufficient to reduce the incidence of papillomas. Papilloma latency increased in both ATS groups, compared to the control value.

After the end of the DMBA treatment, between 86 and 93 days, the average papilloma regressions were 30% for the ATS-14 group, 11% for the control group, and 0% for the ATS-26 group. Changes in the average number of papillomas due to these regressions were apparent in the convergence of papilloma incidences at the control level. After this convergence, papilloma incidences in both ATS groups closely paralleled changes in control incidences (text-fig. 3). Thus ATS administration influenced papilloma development during and immediately after the completion of DMBA treatment. All differences in papilloma incidence resulting from ATS administration were diminished among the 3 groups during the first 2 weeks after the twenty-fifth or last DMBA treatment (text-fig. 3).

The first carcinomas appeared in the ATS-26 group by 86 days, in the control group by 100



TEXT-FIGURE 4.—Papilloma and carcinoma incidences during and after DMBA treatment of sham-operated (control) or splenectomized and/or lymphadenectomized animals. Papilloma: O, control (sham);  $\Delta$ , lymphadenectomized;  $\bullet$ , splenectomized/lymphadenectomized; and  $\blacktriangle$ , splenectomized. Carcinoma:  $\square$ , control (sham);  $\square$  with diagonal lines, lymphadenectomized;  $\blacksquare$ , splenectomized/lymphadenectomized; and  $\blacksquare$  with horizontal lines, splenectomized.

days, and in the ATS-14 group by 114 days. The incidences were 12%, 5%, and 44%, respectively. By 121 days after the first painting, the carcinoma incidence in the ATS groups was approximately 12-fold that of the control (text-fig. 3). This large difference in carcinoma incidence due to ATS was considerably reduced by the end of the experiment (150 days). At this time, only an approximate 20% difference in carcinoma incidence was detectable between the groups (text-fig. 3).

#### *Effects of Regional Lymphadenectomy and/or Splenectomy on Tumor Development.*

Groups of 30 splenectomized, 15 lymphadenectomized, and 15 splenectomized/lymphadenectomized animals were treated with DMBA to study the effect of systemic and/or regional immunosuppression. The control group consisted of 15 sham-operated animals. The results expressed as tumor incidence are shown in text-figure 4.

The results of these experiments were evaluated in terms of papilloma incidence, regression, and latency and of carcinoma incidence and latency; they were correlated with the immunologic status

of animals as influenced by DMBA treatment alone. Text-figure 4 shows the changes in tumor incidence during and after chronic DMBA treatment as modified by surgery before DMBA treatment.

During DMBA treatment of splenectomized or lymphadenectomized animals, the papilloma incidence in both groups increased 15% over the control value, reaching 85% by the last DMBA treatment (86 days). In animals with both their spleen and draining lymph node removed, the incidence of papillomas increased to 100% (a 30% increase above the control level) or twice that of the increase observed for animals with either the spleen or the draining lymph node removed. Papilloma regression during the period between the nineteenth and twenty-fifth DMBA treatments was not sufficient to alter the increase in papilloma incidence (text-fig. 4).

Due to the ratio of papilloma development and regression, a convergence of papilloma incidences after termination of DMBA treatment (between 86 and 93 days) was also observed in this experiment. The resulting similarity in papilloma incidences (between 93 and 100 days) was also apparent in the carcinoma incidences at that time. In parallel

with an expected recovery in cell-mediated immunity, new differences in the observed incidence of papillomas between the groups were established by 107 days (text-fig. 4). This change in papilloma incidences corresponded to a period of increased papilloma regressions. The percentage of regressions was inversely proportional to the observed incidences, *e.g.*, 20% for the lymphadenectomized and 36% for the splenectomized/lymphadenectomized group. Variations in the latency and incidence of carcinomas reflected similar variations in papilloma development. The incidence of carcinomas was increased most significantly (*i.e.*, 93% by 150 days) by the immunosuppressive procedure involving removal of both the spleen and draining lymph node (*see* text-fig. 4; 150 days after the first DMBA treatment).

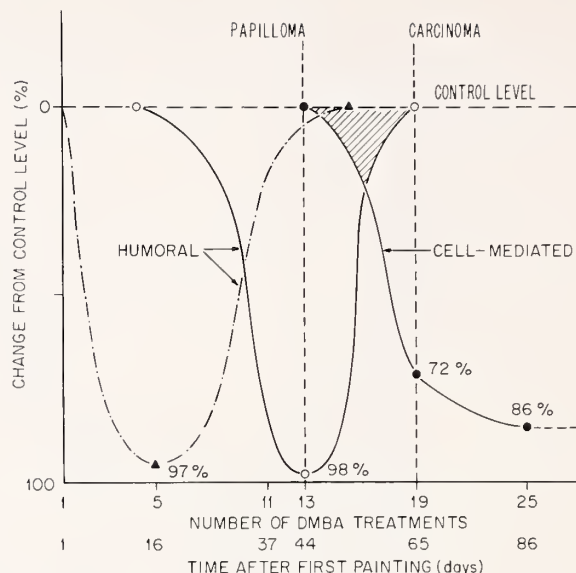
## DISCUSSION

Burnet's theory of immunologic surveillance (21, 22), which implies that suppression of cell-mediated immunity is necessary for tumor development, has been widely adopted as a working hypothesis to study the underlying immunologic mechanism of carcinogenesis. More recently, Prehn (23, 24) hypothesized that a mild degree of antitumor immunity is beneficial to tumor growth. Our observations do not unequivocally support either of these concepts.

Our results show that carcinogen-mediated immunosuppression is actually a *modulation* of both humoral (regional and systemic) and cell-mediated immunity, both of which are functional in tumorigenesis. This modulation of the host's immunity involves not only a quantitative change but also a qualitative one, depending on the ratio of humoral versus cellular immunity.

Such a carcinogen-induced modulation of normal immunity is illustrated in text-figure 5. The profile shows the sequential depression of humoral immunity in the draining lymph node at the site of the DMBA-induced lesion, the depression and recovery of systemic humoral immunity in the spleen and peripheral lymph node, and the subsequent depression of cell-mediated immunity.

The depression of humoral immunity was correlated with the latency and incidence of papilloma and carcinoma development. Papillomas first appeared after the depression of 19S humoral immunity in the ipsilateral draining lymph node, but



TEXT-FIGURE 5.—An idealized diagram illustrating the modulation of normal immunity during chronic DMBA treatment of hamsters. The curves depict depression of humoral (regional and systemic) and cellular immunity as percent change from control levels. Note broken lines indicating the appearance of papillomas and carcinomas. Humoral immunity: ipsilateral lymph node, dotted line; spleen or contralateral lymph node, solid line.

coincidentally with maximal suppression of systemic humoral immunity. This correlation is meaningful only if early papillomagenesis is at least partially controlled by humoral immunity.

The humoral nature of immunologic control of early papilloma development is suggested by the following observations: 1) In animals splenectomized before DMBA treatment to suppress humoral immunity, papilloma and thus carcinoma latency was reduced compared to that in normal and lymphadenectomized animals. 2) Papilloma latency was not reduced by either ATS-14 or ATS-26 used to depress cell-mediated immunity. 3) Present histologic observations of an admixture of plasmacytic and some lymphoid cell infiltrates associated with growing papillae of the early hyperplastic epidermis are in agreement with similar observations of Lappé (25) for 3-methylcholanthrene-treated mouse skin. These functional and histologic observations all suggest that humoral immunity is active in the control of early papillomagenesis. Therefore, it is reasonable to assume that the described transient regional humoral immune depression in the draining lymph node after 5 DMBA treatments and the following depression of systemic



humoral immunity by 13 DMBA treatments are part of the requirements of papilloma development.

Initial papillomagenesis seems contingent on the early suppression of regional and systemic humoral immunity, though cellular immunity cannot be excluded. Papilloma development in the late phase of DMBA treatment appears to be primarily influenced by cellular immunity. The functional role of depressed cellular immunity in papilloma development was defined by Lappé (25), who observed a greater degree of papilloma development on 3-methylcholanthrene-pretreated isografts transplanted to thymectomized and/or X-irradiated recipients than on those transplanted to immunologically intact recipients. Some of our preliminary studies with DMBA-pretreated skin grafts confirm these observations and further suggest that cellular immunity is primarily functional in papilloma survival.

Studies of DMBA-induced development of tumors *in situ* after ATS treatment, splenectomy, and/or lymphadenectomy confirmed the functional role of immunosuppression. The results showed that papilloma and subsequent carcinoma development was favored by an early suppression of humoral and cellular immune reactions.

Woodruff and Smith (26), who studied the effect of ALS (ATS) and normal rabbit serum on tumors, found that considerable amounts of the sera were taken up by tumor cells and observed that, while the antiserum inhibited growth of mammary carcinoma transplants, it enhanced the growth of a chemically induced sarcoma. Because of these observations, we used 2 different regimens of ATS treatment—one for the period preceding tumor development and the other for the period including it. The increase in latency of papillomas in our ATS groups may also be interpreted on the basis of adsorption of the heterologous serum on tumor cells (text-fig. 3). It is suggested that, due to adsorption of ATS, tumor cells are more receptive to immunologic control. This increased receptiveness could also explain the reduced papilloma incidence in the ATS-26 group. In the absence of antiserum adsorption, the papilloma incidence in both ATS groups increased rapidly, probably because of the lack of papilloma regressions and the duration of suppressed cell-mediated immunity.

During the 1st week after the last DMBA treatment, papilloma incidence in the ATS-14 group declined to control levels. This decline was believed

to be caused by a combination of events: a more rapid recovery of cellular immunity due to a shorter ATS regimen and a papilloma regression 20% higher than the control value, possibly because of increased antigenicity of papillomas in response to immunosuppression. Regardless of the dynamics of the regulation of papilloma development, the incidence of carcinomas in the different groups increased proportionally to the extent of ATS treatment.

By the end of DMBA treatment, papilloma incidence in the splenectomized and lymphadenectomized animals, compared to those with only 1 of the 2 organs removed, increased twofold over the control level. The effects of immunosuppression due to the removal of both spleen and draining lymph nodes thus appear to be additive with respect to papilloma incidence by 86 days and to carcinoma incidence by 150 days (text-fig. 4).

That the spleen is functional in regulating papilloma survival after carcinogen treatment is apparent from the differences in papilloma incidences between the splenectomized and nonsplenectomized groups around 107 days after the first DMBA treatment. In the lymphadenectomized and sham-operated control groups, in the presence of the spleen, papilloma incidences remained higher than in the splenectomized groups (text-fig. 4). This interpretation is consistent with the observed recovery of humoral immunity and the theory of "immunostimulation" recently proposed by Prehn (23, 24). The difference in papilloma incidences and number of regressions between the lymphadenectomized and control group also suggests that the draining lymph node, a source of cellular immunity, is functional in papilloma regressions. These differences in papilloma control are reflected in the carcinoma incidences in these groups (107–114 days).

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# Chronic Immunosuppression and Lymphomagenesis in Man and Mice<sup>1, 2</sup>

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**SUMMARY**—Clinical and experimental evidence for the facilitation of the development of malignant lymphomas by coincident persistent antigenic stimulation and chronic immunosuppression was reviewed. The normal average incidence in lymphoreticular neoplasms of 9/100,000 increased significantly from 5–15% in patients with certain immune deficiency diseases, and the frequency of such tumors in human allograft recipients was about 0.4%. In animal models testing specifically the effects of coincident persistent antigenic stimulation with chronic immunosuppression, malignant lymphomas increased from 4% in normal controls to 20 and 60% in experimental animals; whereas depression of humoral immunity could easily be related to lymphoma development, cellular immunity was always abolished in animals developing tumors later. The possible pathogenetic mechanism of immunologic lymphoma development is discussed.—*Natl Cancer Inst Monogr* 35: 183–190, 1972.

THIS presentation deals with etiologic and pathogenetic aspects of immunosuppression and the development of malignant lymphomas. Thanks to the magnificent progress made in virus tumor research, I might be expected to return to the audience and enjoy the ensuing speakers after making only two statements, *i.e.*, malignant lymphoma is a virus-induced disease, and immunosuppression enhances tumor induction by viruses. Being a pathologist, however, I cannot overcome completely my natural conservatism and skepticism because, in general, a variety of insults to the living organism may cause similar patterns of morphologic reaction. Every

tissue has a limited number of reaction patterns and these may be expressed without specific relationship to a given initiating insult. It would be unique in biology if just a single agent, such as a virus, and only this, would be able to cause one specific reaction pattern such as neoplasia. We have been interested for several years in the morphology of immunocompetent tissues during immunostimulation and immunosuppression, and, based on studies correlating morphologic changes with immunologic functions, we have approached the problem of lymphoma development from an angle different from virus research. This approach has led to the concept that the coexistence of persistent antigenic stimulation and chronic immunosuppression is pathogenetically related to malignancy in immunocompetent tissues (1–5). Before discussing this concept further, I will summarize the currently available evidence in support of it.

## DEFECTIVE IMMUNOLOGIC REACTIVITY FOLLOWED BY MALIGNANT LYMPHOMAS IN MAN

A variety of diseases exists in man in which defective immunologic reactivity is accompanied or

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TABLE 1.—Immunosuppression and antigenic stimulation followed by malignant lymphomas, in man

Clinical syndrome	Immunosuppression	Antigenic stimulation	Lymphoma	References
Allotransplantation	Chemical immuno-suppressants, ALS*	Allograft	Reticulum cell sarcoma	(7–10)
Immune deficiency syndromes	Congenital and acquired forms of humoral and/or cellular immune defects	Environmental organisms including pathogens	Malignant lymphoma	See table 2
Autoimmune disorders† (Sjögren's syndrome, rheumatoid disease, Rademacher's disease, etc.)	Defects in cellular immunity, antigenic competition, immuno-suppressive treatment, hypogammaglobulinemia	Autoantigens and/or environmental organisms	Malignant lymphoma, reticulum cell sarcoma	(11–14)
Burkitt's tumor	Defective cellular immunity (?), antigenic competition	Malaria parasites, Epstein-Barr virus		(1, 5, 15)

\* ALS = antilymphocyte serum.  
† Systemic lupus erythematosus, sometimes accompanied by lymphocytic leukemia, should probably be included here.

followed by neoplasia of immunocompetent tissues. All cases where immunosuppression follows lymphoreticular neoplasia as an acquired or secondary phenomenon are intentionally disregarded here because they are not pertinent to the pathogenesis of lymphoma. At the National Library of Medicine, we screened publications for the coexistence of immunosuppression (or of diseases associated with defective immune reactions) and lymphoreticular neoplasia. Of 912 publications fitting these criteria, 311 contained proved or suggestive evidence for immunosuppression preceding lymphoma development. Among the classes of disease accompanied by defective immunologic reactivity were: inherited and symptomatic immune deficiency states, malabsorption syndrome, autoimmune disorders, and immune complex diseases including hepatic cirrhosis, and status post homotransplantation with immunosuppressive chemotherapy. The types of lymphoreticular neoplasms observed encompassed various kinds of malignant lymphoma, thymoma, lymphoproliferative disorders, and chronic lympho-

cytic leukemia. Table 1 summarizes the principal reports of immunosuppression and lymphoreticular malignancies and table 2 indicates frequencies of such neoplasms in immune deficiency syndromes. The incidence of lymphoreticular tumors in patients with demonstrable defects in immunologic reactivity is significantly higher than that observed in the normal population, *i.e.*, 9/100,000 per year, excluding Hodgkin's disease (6).

DEFECTIVE IMMUNOLOGIC REACTIVITY FOLLOWED BY MALIGNANT LYMPHOMAS IN EXPERIMENTAL ANIMALS

Animal experiments for the induction of malignant lymphomas and leukemias have been done for many years, by a variety of procedures. Essentially all of these, with the use of chemical carcinogens, ionizing radiation, or viruses, induce some degree of immunosuppression whether intended or not. Weak immunosuppressants must be administered to immunologically incompetent animals to

TABLE 2.—Immune deficiency syndromes and lymphoreticular neoplasms

Syndrome	Lymphoma	Estimated incidence (%)	References
Swiss-type agammaglobulinemia	Malignant lymphoma, thymoma, lymphocytic leukemia	5	(16, 17)
Wiskott-Aldrich syndrome	Malignant reticuloendotheliosis, malignant lymphoma, acute leukemia, malignant reticulosis	10–15	(17, 18)
Ataxia telangiectasia	Malignant lymphoma, acute lymphocytic leukemia, acute lymphoblastic leukemia	10	(17, 19–23)
IgA-deficient sprue	Intestinal lymphoma, malignant reticulosis	7	(24, 25)
Acquired hypogammaglobulinemia	Lymphosarcoma	–10	(17, 26–28)

TABLE 3.—Experimental lymphoma induction by coincident immunosuppression and antigenic stimulation

Experimental design	Immunosuppression	Antigenic stimulation	Lymphoma	References
Chronic allogeneic disease and wasting	Chronic graft-versus-host reaction, amethopterin, runting, thymectomy	Parental spleen cells into F <sub>1</sub> hybrids, spleen cells across a weak histocompatibility barrier, chronic bacterial endotoxemia, environmental organisms	Reticulum cell sarcoma, lymphocytic lymphoma, thymic lymphoma, lymphoreticular sarcoma, reticuloendotheliosis	(29–34)
Chronic autoimmune disorders	Azathioprine, thymectomy, antigenic competition, relative and absolute immunologic defects	Autoantigens, environmental organisms	Malignant lymphomas, lymphoreticular sarcomas, lymphoblastic lymphosarcomas	(35–37)
Tumor xenografting or allografting	Specific tolerance	Tumor antigen, histocompatibility antigen	Malignant lymphoma, lymphoblastic lymphoma	(38, 39)
Virus lymphoma induction	Immunologic immaturity of recipient immunosuppressive effect of virus, antigenic competition	Virus antigen, environmental organisms	Stem cell lymphoma, lymphoblastic lymphoma, lymphocytic leukemia	(40)

induce leukemia or lymphoma, particularly with murine leukemia viruses which are rather ineffective when given to normal adult animals. Table 3 summarizes various experimental models for the induction of malignant lymphoma that most obviously include immunosuppressive mechanisms. In all the experiments cited, the incidence of induced lymphoreticular neoplasms is significantly above the spontaneous incidence of such tumors in the respective strain and species. In most, the latent period is also significantly shortened when compared to the age of the normal animals at the onset of spontaneous neoplasms. In some, even the histologic type and the site of development of induced lymphomas differ from the spontaneous ones. These differences can be observed despite the widespread distribution of "leukemogenic" C-type

RNA viruses, and despite the high probability that such viruses were present, active or occult, in all animals with induced lymphomas.

Therefore, we concentrated on a series of experiments on the immunologic reactivity of mice during the latent period of lymphoma induction (2, 3, 5, 47), and used the model of persistent antigenic stimulation coexistent with chronic immunosuppression (table 4). This model was deduced from the above-mentioned clinical and experimental data. Various nononcogenic substances served as antigens and immunosuppression was attempted with several compounds in everyday clinical use, none of which is overtly oncogenic. The high incidence of malignant lymphomas induced by this procedure is obvious in table 4. The type of tumor was always a systemic thymus-derived T-cell lymphoma.

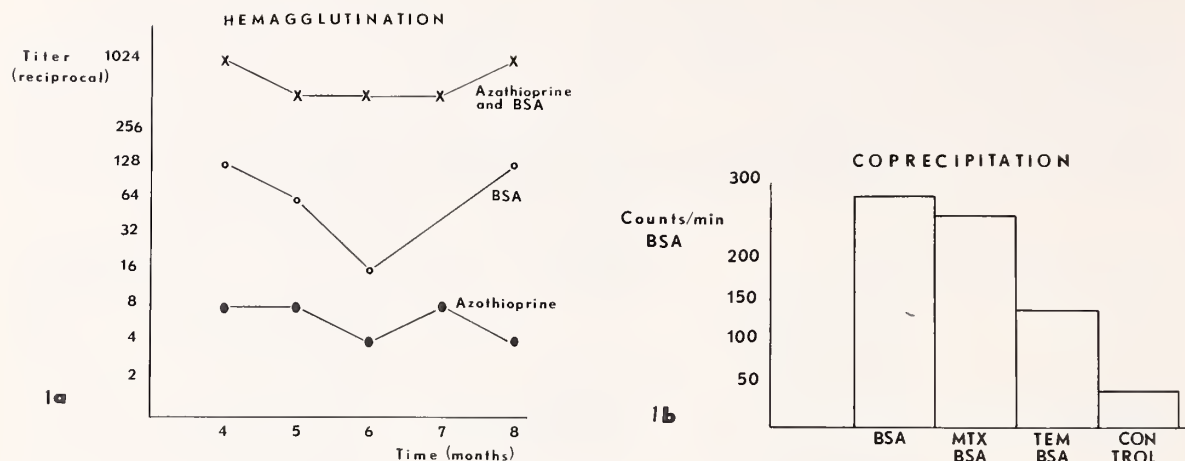
TABLE 4.—Incidence in immunologically induced mouse lymphomas\*

Mouse strain	Number of mice	Immunosuppression	Antigenic stimulation†	Lymphoma incidence (%)	
				Experimental	Controls
BALB/c	32	Azathioprine	LDV†	32	0
	32	Azathioprine	TB	20	0
	64	Azathioprine	BSA	20	0
	32	ALS	LDV	0	0
	32	ALS	TB	0	0
DBA	34	Azathioprine	BSA	66	12‡
	34	Azathioprine (low)	BSA	16	3‡
	34	Methotrexate	BSA	12	0
	34	Triethylene melamine	BSA	0	0

\* From (5).

† ALS = anti-lymphocyte serum; LDH = lactic dehydrogenase elevating virus; TB = tubercle bacteria in adjuvant; BSA = bovine serum albumin.

‡ Immunosuppression alone.



TEXT-FIGURE 1.—Immune response of experimental mice during the latent period of lymphoma development. *a*) Increase in anti-BSA antibodies in BALB/c mice which later had lymphomas (x). *b*) Average titers during the latent period of anti-BSA antibodies in DBA mice; controls received immunosuppressants only. In all BALB/c and DBA mice treated with BSA and immunosuppressants, or with immunosuppressants alone, the cellular immune response was completely abolished, as controlled by skin testing [text-figs. reproduced from (5)].

phoma, as characterized by the demonstration of theta antigen on tumor cells (41). The immunologic reactivity against the inducing antigen in our model is summarized in text-figures 1A and 1B. Antibody measurements during the latent period of lymphoma development and in early lymphoma cases by passive microhemagglutination assay and the coprecipitation method. Cellular immunity was evaluated by skin test with the inducing antigen (41). As table 4 indicates, cellular immunity was completely absent in all animals that developed lymphoma later, whereas humoral immune reactions remained variable.

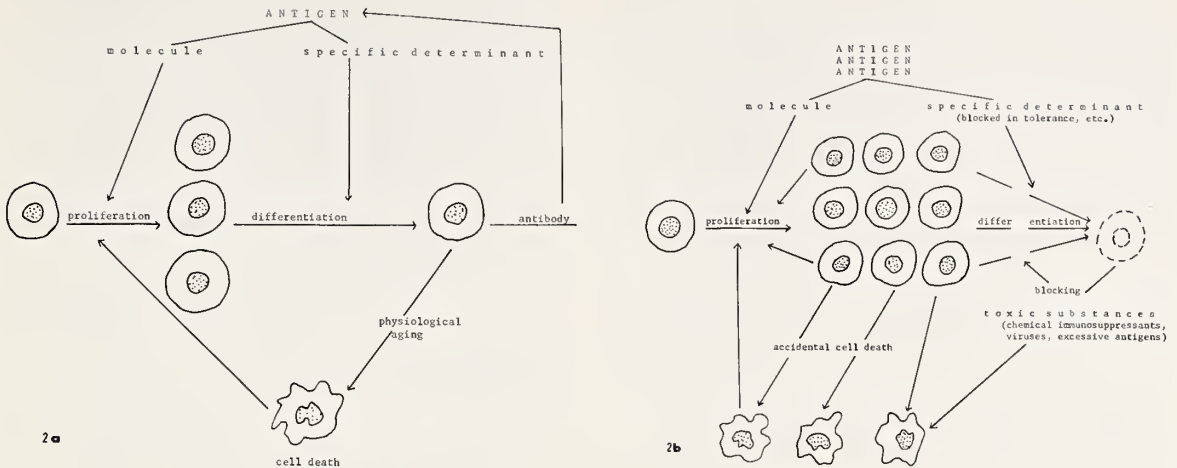
## THEORETICAL CONSIDERATIONS CONCERNING PATHOGENESIS OF MALIGNANT LYMPHOMAS

The experiments appear to confirm the importance for lymphoma development of a defective immune response to a persistently stimulating antigen. Leukemia viruses, from this assumption, combine 2 necessary characteristics: they represent a continuously present and even replicating antigen, and they have immunosuppressive potencies (42–45). Since these viruses are ubiquitous in nature, the relatively low-incidence of spontaneous tumors remains unexplained; perhaps a second competitive antigenic stimulation is necessary such as malaria infection in Burkitt's tumor (5). "Nononcogenic" viruses, even in persistent infections, may

need the addition of an immunosuppressant to induce lymphoma, *e.g.*, our experiments using the LDH virus in combinations with azathioprine. Antigenic substances other than viruses appear to be active as well, provided that they are persistently present and combined with chronic immunosuppression. Tolerance induction in this situation does not alter the consequences, since it does not necessarily inhibit proliferation of stem cells in immunocompetent tissues (46, 47). In acquired or congenital immune deficiency diseases of man, well-documented recurrent and chronic infections can provide the persistent antigenic stimulation necessary for lymphoma development. In human allograft recipients, the combination of immunosuppression, to avoid transplant rejection with antigenic stimulation by the transplant itself, causes lymphoreticular neoplasms in 0.4% of the cases (9).

The increased incidence of malignant lymphomas in autoimmune diseases or immune complex diseases is less easy to explain. Persistent antigenic stimulation certainly is present in all of these. Immunologic defects, except for cases treated with chemical immunosuppressants, are difficult to demonstrate. In systemic lupus erythematosus, a defect in cellular immunity probably exists (48). Theoretically, however, there are several mechanisms by which relative immunologic nonresponsiveness could be produced: 1) A state of tolerance against circulating antigen-antibody complexes





TEXT-FIGURE 2.—Physiology and pathology of antigen-induced lymphoreticular cell proliferation. *a*) Physiologic conditions; *b*) pathologic conditions preceding the development of malignant lymphoma.

inhibits further antibody production but does not inhibit cell proliferation. 2) Continuous high-level autoantigenic stimulation competes with antigenic stimulation by the normal exogenous agents and inhibits proper antibody formation against these; nevertheless, these agents still evoke cell proliferation. 3) Some evidence exists for a physiologic balance between humoral and cellular types of immune responses (49–51); therefore, enhancement of one type could be accompanied by at least relative depression of the other type. In many autoimmune diseases the humoral immune response is obviously enhanced. 4) Immunologic hyperresponsiveness may be followed by immunologic hyporesponsiveness, *i.e.*, exhaustion, which then precedes lymphoma development. At present, it is impossible to prove whether any of these mechanisms is indeed acting, and much more work is needed to characterize the immunologic defect preceding lymphoreticular neoplasia. The problem is complicated by the fact that any defect may be quite specific toward just one or a few antigens, none of which is readily recognized.

Radiation and chemical carcinogens used for experimental lymphoma induction are both immunosuppressants, and normal everyday antigenic stimulation is probably a sufficient complementary factor to initiate cell proliferation. Although several investigators have induced lymphomas by persistent antigenic stimulation alone or by immunosuppression alone (52–55), they cannot exclude coincident antigenic stimulation.

In discussing the pathogenesis of malignant lymphomas after coexistent persistent antigenic stimulation and chronic immunosuppression, the general biology of cell proliferation and differentiation must be considered. Cell proliferation in general is initiated by the necessity of repair or of increase in cell function above a certain level. Accordingly, both toxic substances and antigens can initiate stem cell proliferation in the lymphoreticular tissues. The response is sometimes dose-dependent, since after severe toxic insults, excessive necrosis follows without much regenerative cell proliferation, and mild antigenic stimulation probably can be followed by hypertrophy (*i.e.*, cellular activation) rather than by hyperplasia (*i.e.*, cell proliferation). Cell proliferation following toxic cell death is thought to be caused by release of intracellular stimulatory substances or by the abolishment of a physiologic proliferation-inhibition (56–59). Cell proliferation after antigenic stimulation may follow similar pathways, although cell functional inhibition rather than cell death may be the initial stimulatory effect. Also, the antigen, once gaining access to specific receptor cells of the immunocompetent tissue, probably stimulates their proliferation and function without primary inhibition. Cell differentiation occurs in response to the need for a specific cell function in cells that possess the genetic anlage to develop it. A typical example of this mechanism is the phenomenon of enzyme induction (60, 61). The antigen molecule as such appears to be an adequate stimulus for the

proliferative response of lymphoreticular cells, whereas the specific antigenic determinant directs differentiation of immunoglobulin-producing cells and induces synthesis of specific receptor sites on antibody molecules. The ability to synthesize immunoglobulins is genetically pre-encoded in immunocompetent stem cells (62). This differential function of the antigen molecule and of its specific determinant explains why cell proliferation continues in immunocompetent tissues in immune complex diseases or in tolerant individuals, even though specific antibodies may not be adequately synthesized. Moreover, in our model and in certain human cases such as homograft recipients, immunosuppressive chemicals interfere with cell differentiation (63, 64).

In summary, from these theoretical considerations, the biological effect on lymphoreticular tissues of coexistent persistent antigenic stimulation and chronic immunosuppression is summarized in text-figures 2a and 2b. It can be seen that the mechanisms stimulating cell proliferation are markedly in excess of those inducing cell differentiation. This condition leads to marked progressive hyperplasia. It was suggested by Linzbach (65) and others (66, 67) that a certain degree of imbalance between cell proliferation and cell differentiation supports further cell proliferation. In our model, excessive stimulation of cell proliferation can induce a vicious cycle of essentially autonomous cell proliferation, *i.e.*, malignant lymphoma.

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# Some Effects of Age on Immunity and Chemical Oncogenesis in Rats<sup>1,2</sup>

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**SUMMARY**—The immune responses of female and male Wistar rats, 1, 12, and 18 months old, were measured at various times during and after treatment with the oncogen 3-hydroxyxanthine, to determine whether age-associated immune competency during injection of the oncogen was related to eventual tumor incidence. Hemagglutinin antibody formation was approximately the same in both sexes and in all age groups and was not affected by the oncogen. Sarcoma-180 grafts generally grew larger in males; in most cases, the larger the tumor graft the older the host. Twelve months after the initial dose of 3-hydroxyxanthine, cumulative tumor incidence, based on population at risk, was highest in the males of the 18-month-old group and lowest in the multiparous females of the 18- and 12-month-old groups. As of the present, data for the oldest male group only suggest a positive relationship between tumor incidence and cellular immune competency at the time of administration of oncogen.—*Natl Cancer Inst Monogr* 35: 191–196, 1972.

LAW (1) and Allison and Taylor (2) reported a substantial incidence of tumors in normally resistant mice thymectomized and exposed to “room” infection with polyoma virus. It was concluded that consequential viral tumorigenesis was the result of an inadequate response by the immunologically impaired mice to the “natural” infection. Since impairment of immunologic competence does occur in aged individuals (3–7), one may postulate similar neoplastic consequences in an aged population exposed to an oncogen. It should be possible, then, to determine experimentally whether a natural diminution of immune competency influences tumor incidence. In the present experiments, such

information was sought in randombred rats of various ages treated with a chemical oncogen.

## MATERIALS AND METHODS

**Rats.**—Randombred Wistar females and males (CFN, Carworth, New City, N. Y.) were obtained as weanlings or retired breeders. They were bedded on wood shavings in stainless-steel cages, fed Purina Chow pellets, given water *ad libitum*, and maintained at about 24°C. Rats, 1, 12, or 18 months old at the beginning of the experiment, retained the same age designations for the duration of the experiment for ease of identification.

**Oncogenesis.**—The oncogen, 3-hydroxyxanthine (8), was prepared as a homogenized suspension in 0.5% carboxymethylcellulose in physiologic saline (CMC). Rats were given, 3 times weekly for 8 weeks, subcutaneous injections in the interscapular area of 0.1 mg of 3-hydroxyxanthine in 0.5 ml CMC. This dose results in an approximate 50% tumor incidence at the site of injection in 18 months

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when administered to young-adult (1–2 months old) rats. Controls received 0.5 ml CMC.

Rats were palpated bimonthly for tumors beginning the 3d month of the experiment. Tumor latency was designated as the time in months between administration of the first dose of oncogen and palpation of a nodule at the site of injection. All rats were examined at autopsy, and suspicious masses were examined microscopically.<sup>4</sup> Tumor incidence was presented as a ratio: the number of rats with induced tumor/the total number of rat years at risk.<sup>5</sup>

*Immune response.*—Hemagglutinin antibody to sheep red blood cells (SRBC) was measured by a modified procedure of Stravitsky (9) with the use of a Microtiter apparatus (Cooke Engineering, Alexandria, Va.). Rats were immunized with a phosphate-buffered (pH 7.2) saline suspension containing  $1 \times 10^9$  cells/ml at a dose of 0.01 ml/g body weight and they were bled by tail snipping 14 days later. The serum was stored at  $-20^\circ\text{C}$  and before being tested it was diluted with Veronal-buffered gelatin (40 ml Veronal buffer, pH 7.2, 10 ml of a 2% gelatin solution, plus 150 ml deionized water). A suspension of SRBC containing  $1 \times 10^8$  cells/ml of Veronal-buffered gelatin was added to duplicate, serial twofold dilutions of heat-inactivated serum, and the Microtiter plates were incubated for 2 hours at room temperature. Titers were reported as reciprocals of the last dilution ( $\log_2$ ) at which a  $\pm$  activity was recorded.

Mouse Sarcoma 180 (S180) was used for the measurement of graft-rejection response. Rats were implanted subcutaneously in the flank with 0.5 ml of a 50% suspension of minced, 7-day-old, mouse-grown S180. Length, width, and depth of the subcutaneous masses (growths) at the site of implantation were measured on alternate days for 2 weeks, starting on the 7th day. Previous experiments showed no maxima before this time. Growths were reported as volumes calculated from the equation  $V = 4/3\pi(r_1r_2r_3)$ , wherein length/2 =  $r_1$ , width/2 =  $r_2$ , and depth/2 =  $r_3$ . Growth volumes were compared on the basis of the maximum size attained during the period of measurement; most

maxima occurred on the 7th day, and only a few on the 9th day.

*Experimental design (table 1).*—Three main groups (A, B, and C) contained rats of both sexes and 3 age groups, 1, 12, and 18 months old. Each group was composed of oncogen-treated and CMC-treated (control) rats. Group A consisted of rats treated only with oncogen or CMC and then kept to ascertain tumor incidence. Group B was treated like group A and tested for hemagglutinin antibody. Group C was treated similarly and tested for rejection of S180 grafts. Data for tumor incidence do not include information from groups B and C because of their special treatments.

## RESULTS

### Immune Response

*Antibody titers.*—Groups of 1-, 12-, and 18-month-old rats of both sexes were immunized after the third injection (5th day) or after the twenty-fourth and last injection (54th day) of 3-hydroxyxanthine. Control groups consisting of male and female rats of the above ages given injections of CMC alone were immunized on the 54th day. All rats were bled 14 days after immunization, and serum hemagglutinin antibody titers were determined.

Twelve nonimmunized, untreated rats, 2 of each sex in each age group, were tested for natural SRBC hemagglutinin antibody for baseline data. Titers were 0 in 8, 1 in 3, and 2 in 1 rat. Table 2 shows the results for the oncogen-treated and control rats. Since little difference was found between titers of rats immunized on different days or between sexes, these data were combined. No significant differences were seen between mean titers for treated and control groups, nor among antibody titers for the 3 age groups. Therefore, apparently neither age, sex, or treatment with 3-hydroxyxanthine influenced hemagglutinin antibody formation.

*Graft response.*—Immune responses to grafts of S180 were determined in rats, 1, 12, and 18 months old, administered 3-hydroxyxanthine or CMC. Volumes of the growths appearing at the site of injection were calculated. The medians of the maximum volumes in each group are presented in table 3. Medians for tumor growth generally increased with host age and were usually larger in the males.

The greater amount of tumor growth with increasing age was more manifest when the number

<sup>4</sup> Tissue diagnoses by Dr. J. M. Budinger are gratefully acknowledged.

<sup>5</sup> The invaluable discussions with Dr. Isabel M. Moun-tain, Statistician, are appreciated.



TABLE 1.—Experimental design

Age group (months)	Number of rats		Treatment*	Immunologic test	
	♀	♂		Type	Time initiated (day)†
A	18	34	36	Oncogen	None
	12	20	20	"	"
	1	20	20	"	"
	18	10	10	Control	None
	12	10	10	"	"
	1	10	10	"	"
B	18	5	5	Oncogen	SRBC‡
		5	5	"	"
	12	5	5	"	"
		5	5	"	"
	1	5	5	"	"
		5	5	"	"
	18	5	5	Control	"
	12	5	5	"	"
	1	5	5	"	"
C	18	5	5	Oncogen	S180§
		5	5	"	"
	12	5	5	"	"
		5	5	"	"
	1	5	5	"	"
		5	5	"	"
	29	4	5	Control	"
	18	5	5	"	"
	12	5	5	"	"
	1	5	5	"	"

\* Oncogen: 0.1 mg 3-hydroxyxanthine administered subcutaneously, 3 times a week for 8 weeks. Control: 0.5 ml CMC (see "Materials and Methods") administered by same schedule as that used for the oncogen.

† Day 0 = day of first injection of oncogen; day 5 = day after the third dose of oncogen; day 54 = day after the last dose of oncogen.

‡ SRBC hemagglutinin antibody.

§ Subcutaneous graft of 0.5 ml of a 50% suspension of mouse S180.

of growths  $\geq 1.5 \text{ cm}^3$  was considered. For such a comparison, data for both sexes were pooled, as were those for the oncogen-treated rats grafted at different times but within the same age groups. Results are shown in table 4.

No statistical difference was noted between the numbers of large growths in the different CMC-treated age groups. However, there was a signifi-

cant trend [ $P \leq 0.05$  (10)] toward more such growths with increasing age. In the oncogen-treated rats, where the populations were larger, there were significantly more growths  $\geq 1.5 \text{ cm}^3$  in the 18-month-old rats compared with the 12- or 1-month-old rats ( $P \leq 0.01$ ). There was no difference between the CMC-treated and oncogen-treated age groups.

TABLE 2.—Influence of age on immune response to SRBC

Age group (months)	Mean antibody titer* $\pm$ sd†			
	Controls		Oncogen-treated‡	
	Number of rats	Titer	Number of rats	Titer
1	10	9.0 $\pm$ 0.8	18	7.4 $\pm$ 1.4
12	10	8.7 $\pm$ 1.4	19	8.7 $\pm$ 1.3
18	9	7.6 $\pm$ 1.5	20	8.8 $\pm$ 1.2

\* Reciprocal of serum dilution, log<sub>2</sub>.

† sd = standard deviation.

‡ 3-Hydroxyxanthine given subcutaneously at a dose of 0.1 mg, 3 times a week for 8 weeks.

TABLE 3.—Influence of age and treatment on subcutaneous growth of S180 in rats

Treatment*	Sex	Age groups (months)†			
		1	12	18	29
CMC	♀	0.4‡	0.6	1.6	1.9
	♂	1.5	0.9	1.6	2.6
3-Hydroxyxanthine (day 0)	♀	0.2	0.6	0.9	
	♂	0.2	0.8	1.3	
3-Hydroxyxanthine (day 54)	♀	0.8	0.7	0.7	
	♂	0.2	0.9	2.4	

\* See "Materials and Methods."

† Five rats per sex per age group (4 in 29-month-old female group).

‡ Median of maximum volumes (cm<sup>3</sup>) attained by tumor implants during the first 7–9 days.

### Tumor Induction

Male and female rats of the 3 age groups were simultaneously given subcutaneous injections of 3-hydroxyxanthine. They were examined periodically for tumors at the site of injection. Since the survivors continuously declined due to deaths from tumor and other causes, cumulative tumor incidence was presented on a population-at-risk basis. (See "Materials and Methods" for explanation of the measurement.) Table 5 shows the results of the first 12 months of this experiment, which is being continued for the lifespan of the rats. Tumor appearance was unusually delayed, and tumor incidence was low in the 12- and 18-month-old females. In the other male and female age groups, the latencies for the first tumors were as expected for a dose yielding a 50% tumor incidence in 18 months. However, tumor incidence was greatest in the 18-month-old males. The ratio 0.31 indicates that, at 12 months after the initial dose of 3-hydroxyxanthine, approximately 1 of 3 rats per rat year at risk should have had an induced tumor.

TABLE 4.—Effect of age on proportion of S180 growths  $\geq 1.5$  cm<sup>3</sup> in rats

Age group (months)	CMC controls	3-Hydroxyxanthine-treated
1	4/10	3/20
12	3/10	3/20
18	6/10	11/21
29	8/9	

### DISCUSSION

In a continuing investigation of the relation of aging to immunity and oncogenesis, immune responses of rats of various ages were ascertained to determine whether the immune competency of the host at the time of oncogenic insult was related to eventual tumor incidence. Because many oncogens are immunosuppressants, immune reactivities were measured before, during, and after administration of the oncogen. Thus, sometimes the rats were 8 weeks older at the time of testing than implied by the age-group designation. Past and present results indicated that this minor age differential has little effect.

In rats given 3-hydroxyxanthine in a single dose of 7 mg or multiple doses of 3–6 mg, immunodepressive activity was only moderate (17). The total

TABLE 5.—Influence of age on cumulative incidence of chemically induced\* subcutaneous tumors in rats. Data are presented as a ratio: Number of rats with tumors/total rat years at risk

Time in experiment (months)	Ratios of tumor incidence					
	Age group (months)					
	18 ♂ (36)†	18 ♀ (34)	12 ♂ (20)	12 ♀ (20)	1 ♂ (20)	1 ♀ (20)
7	0	0	0	0	0	0.09
8	0.09	0	0.15	0	0	.08
9	.18	0	.14	0	0.07	.14
10	.21	0.04	.19	0.07	.07	.19
11	.24	.04	.18	.06	.18	.17
12	.31	.04	.16	.06	.17	.16

\* 3-Hydroxyxanthine given subcutaneously at a dose of 0.1 mg 3 times a week for 8 weeks.

† Numbers in parentheses indicate number of rats in the group at beginning of experiment.

dose of 2.4 mg used in the present experiments had no such effect on hemagglutinin antibody formation (table 2).

The term "growth," describing the masses appearing at the site of S180 implantation, indicated visually unconfirmed tumor growth. We previously reported confirmed S180 growth averaging 1.3 g in 43% of weanling rats (12). However, more recent data (17) revealed a lower incidence of takes, but growths approximating 1.5 cm<sup>3</sup> appeared to be viable tumor tissue. In the present experiments, the occurrence of masses  $\geq 1.5$  cm<sup>3</sup> in 4/10 rats 1 month old was unusual (table 4). All (4/5) were in males and none (0/5) in females. However, the in-

creased number of S180 tumors this size in the old rats was a presumptive indication of an impaired rejection response. This impairment was not complete, because all tumors were rejected eventually.

Neither sex nor age influenced antibody response in these rats, as was reported for mice (3, 13), but both factors affected cellular immune response and tumor incidence. Heterologous tumor growth generally increased with increasing age (table 3), suggesting a concomitant decrease in rejection response. Also, growth of S180 was generally greater in the males in most age groups. In regard to susceptibility to oncogenesis, the high tumor incidence anticipated for the oldest male and female groups did not entirely materialize. Twelve months after injection of the first dose of oncogen, cumulative tumor incidences were very low in the groups of females 18 and 12 months old (table 5). The possibility that the multiparous condition of these retired breeder rats had some effect on susceptibility to tumor induction needs further investigation.

The 12-month experience also showed that the highest tumor incidence occurred in the 18-month-old males. This result is only suggestive of a relationship among age at exposure to oncogen, deficient cellular immune response, and susceptibility to tumorigenesis. However, other unconfirmed data indicate an important temporal relationship between oncogenic insult and immune status (14). A single dose of cortisone given with the first, but not with the tenth or sixteenth, injection of oncogen increased the incidence of early tumors. Perhaps these critical interrelationships could be further clarified by a quantitative comparison between the degree of impairment of host immunity and susceptibility to tumor induction.

An immunologic surveillance mechanism postulated by Thomas (15) and expounded by Burnet (16) is believed to function in neoplastic disease. Good (17) recently reviewed many desiderata favoring the existence and importance of such a mechanism. Prehn (18, 19) suggested caution in consideration of the weak effectiveness of this mechanism. However, the importance and strength of immune surveillance become evident when the immune mechanism is absent (6) or curtailed (17). We recently obtained additional positive evidence for increased tumor incidence and decreased latency in experiments in chemical oncogenesis. Small doses of 3-hydroxyxanthine yielding low tumor incidences induced 2 and 3 times as many

tumors when the host was immunodepressed by administration of cortisone acetate (14). Thus an age-related immune deficit becomes important, especially when the impairment involves the cell-mediated response believed to be most involved in the control of neoplasia. Intensive investigation of oncogenesis from the viewpoint of age-related immune deficiency should be fruitful in developing more positive means for the control of neoplasias.

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# Spontaneous Regression of Autochthonous Malignant Lymphomas Induced in Swiss and NZW Mice by 1-Ethyl-1-Nitrosourea<sup>1</sup>

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**SUMMARY**—A single injection of the carcinogen 1-ethyl-1-nitrosourea (ENU), in the order of 1  $\mu$ mole/g body weight, induced malignant lymphomas in very high frequencies in young-adult mice of many strains, e.g., Swiss, NZW, AKR, DBA/2, and C57BL. In certain strains only, among them NIH General Purpose (Swiss) and NZW, a variable proportion of stem cell lymphomas or poorly differentiated lymphocytic lymphomas induced by ENU spontaneously regressed. Regression was characterized histologically by disintegration of neoplastic cells, accumulation of macrophages in tumor tissue, and subsequent replacement of spleen, lymph nodes, thymus, and visceral lymphoma deposits by non-neoplastic, fibrous connective tissue. Regression was observed in lymphomas of both thymic and extra-thymic origin and, under certain conditions, was observed in as many as 12–20% of lymphomas. Lymphomas which subsequently regressed could be transplanted during the initial florid phase of the disease, but not after the regression phenomenon appeared histologically complete. Regression was accompanied by intractable aplastic anemia, affecting both spleen and bone marrow and ineffectively compensated by myeloid metaplasia in other organs, to which affected animals eventually succumbed.—*Natl Cancer Inst Monogr* 35: 197–209, 1972.

THE MOST common forms of lymphoreticular neoplasia in the mouse are malignant lymphomas of the lymphocytic or the undifferentiated lymphoid cell varieties. These lymphomas frequently originate in an involuted thymus (1), where they grow rapidly to produce a mediastinal tumor. Neoplastic cells disseminate rapidly and widely throughout the body, replacing the normal archi-

tecture of the spleen and lymph nodes and producing perivascular deposits in thoracic and abdominal viscera. These neoplasms occur in varying frequencies in different strains and are especially common in strain AKR. They can be induced by a wide variety of chemical carcinogens, by ionizing radiation, and by certain strains of murine leukemia virus, most notably Gross and Moloney leukemia viruses and the radiation leukemia virus of Kaplan (2).

Generally, these neoplasms are rapidly fatal and are characterized histologically by profusely cellular, rapidly growing tumor deposits. Recently we

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<sup>2</sup>National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

reported what is apparently the first observation of spontaneous regression of these neoplasms, accompanied by aplastic anemia (3). This phenomenon occurred in certain strains of mice in which undifferentiated and lymphocytic lymphomas were induced by 1-ethyl-1-nitrosourea (ENU). Regression was characterized histologically by the disintegration of neoplastic cells in tumor deposits, a massive increase in the number of "starry-sky" macrophages within these deposits, and eventual fibrosis and scar formation when the neoplastic cells had been eradicated. This paper describes the pathogenesis of the condition, its occurrence in various strains of mice, and its possible relationship to certain other syndromes observed in both human and animal disease.

## MATERIALS AND METHODS

**Chemical.**—The carcinogen ENU was synthesized and administered to mice as previously described (3).

**Animals.**—Randombred NIH General Purpose (GP) Swiss-Webster mice and inbred strains AKR/N and NZW/BIN were obtained from the Rodent Production Section, Division of Research Services, National Institutes of Health. Inbred Swiss-Webster mice, derived from the randombred GP line and designated NGP/N, were provided by

Dr. Carl Hansen, Head of the Genetics Unit in the Rodent Production Section, who developed this strain by brother X sister matings through more than 20 generations. We tested these animals for genetic homogeneity; skin homografts were accepted and retained permanently.

**Histology.**—All tissues, including abdominal and thoracic viscera, sternum, and peripheral lymph nodes, were fixed in 4% unbuffered aqueous formaldehyde. They were embedded in paraffin and stained with hematoxylin and eosin, periodic acid-Schiff, van Giesson, methenamine silver, Ziehl-Nielson, methyl green-pyronine, and various silver impregnation methods for reticulin, including those of Bielschowsky, Wilder, and del Rio Hortega.

## RESULTS

### Induction of Malignant Lymphoma in Mice by ENU

Malignant lymphomas of various types were readily induced in many strains of mice by a single exposure to ENU, at a dose of 0.5  $\mu$ mole/g body weight or more, administered either transplacentally or when the mice were 5–6 weeks old. Both sexes were susceptible. Most of these malignant lymphomas were lymphocytic or undifferentiated (stem cell) and frequently originated in the thymus. A few reticulum cell neoplasms, types A and

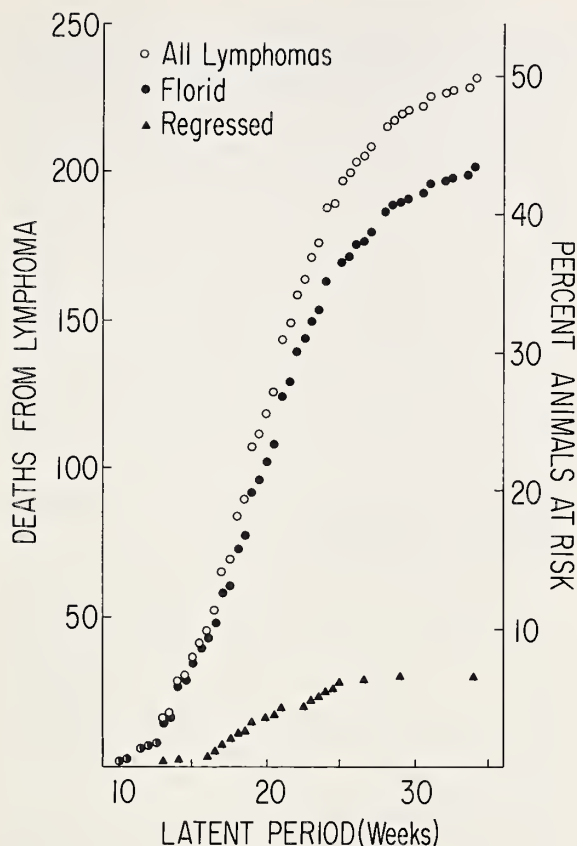
TABLE 1.—Incidence of malignant lymphoma and frequency of regression within 1 year after treatment in different strains of mice given a single intraperitoneal injection of ENU at 5–6 weeks of age

Strain	Sex	Number	ENU dose ( $\mu$ mole/g)	Malignant lymphomas, all forms (%)	Average latency period (wk)*	Regressions†		
						Incipient (%)	Partial (%)	Complete (%)
GP	F	462	1.0	246 (53)	20.9	18 (7.3)	15 (6.1)	15 (6.1)
GP	F	52	0	2 (4)	51.8	0	0	0
NGP/N	F	67	1.0	55 (82)	20.9	4 (7)	2 (7)	1 (2)
NGP/N	F	53	0	1 (2)	36.5	0	0	0
NGP/N	M	51	1.0	29 (57)	21.4	1 (3)	1 (3)	1 (3)
NGP/N	M	55	0	0				
NZW/BIN	F	104	1.0	94 (90)	14.7	3 (3)	4 (4)	3 (3)
NZW/BIN	F	69	0.75	56 (81)	16.1	0	0	0
NZW/BIN	F	24	0	1 (4)	31.0	0	0	0
NZW/BIN	M	95	1.0	87 (92)	16.9	5 (6)	3 (3)	1 (1)
NZW/BIN	M	100	0.75	75 (75)	18.5	2 (3)	2 (3)	1 (1)
NZW/BIN	M	29	0	0				
AKR/N	F	30	1.0	27 (90)	19.9	0	0	0
AKR/N	F	20	0.5	17 (85)	24.2	0	0	0
AKR/N	F	50	0	44 (88)	31.8	0	0	0

\* Interval between ENU or vehicle injection and death. Controls were given solvent (trioctanoin).

† Percentages (in parentheses) indicate the proportion of cases of lymphoma in which the regression syndrome developed.





TEXT-FIGURE 1.—Development of malignant lymphomas and of regression in a group of 462 female GP mice given 1  $\mu$ mole ENU/g body weight at 5 weeks of age. Latency period is the interval between ENU treatment and death. The curve for regressed lymphomas includes instances of complete or partial, but not of incipient, regression.

B, granulocytic leukemias, and (very rarely) erythroblastic and plasma cell leukemias were also induced. Both the total incidence and the latency period varied markedly from strain to strain (table 1).

Lymphoma regression was observed only in strains GP, NGP/N, and NZW/BIN. Of these strains, the one in which this phenomenon occurred in the highest incidence and in which the regression was most complete was the randombred GP line. All lymphomas that regressed were of the undifferentiated or lymphocytic types. The development of lymphomas and of regression in a population of ENU-treated GP mice is shown in text-figure 1.

### Pathogenesis of Lymphoma Regression

The first physical sign of the onset of regression was the rapidly increasing pallor resulting from anemia, which was easily observed in the 3 responsive albino strains. Subsequent physical signs included a rapid decrease in severity of the exophthalmos and labored, diaphragmatic breathing caused by a mediastinal (thymic) mass. Often one could also detect a steady, day-to-day decrease in the size of the previously palpable spleen and enlarged peripheral lymph nodes. As the anemia became more severe (the hematocrit sometimes dropping to 2–4%), the animals became more cachectic and lost weight progressively until they succumbed to sepsis and anemia. At autopsy, anemic mice in complete regression no longer had any grossly detectable signs of malignant lymphoma. Their viscera were pale but of normal size; lymph nodes and thymus were smaller than normal and deep yellow or orange.

Lymphocytic and stem cell lymphomas induced by ENU in these strains of mice were characterized during their florid stage by the presence in tumor deposits of large macrophages with clear cytoplasm, which contained phagocytosed red blood cells and nuclear debris from disintegrating lymphoid tumor cells. These macrophages resembled the “starry-sky” macrophages of Burkitt’s lymphoma in man (4) and the tingible-body macrophages seen in germinal centers of normal lymph nodes. As regression proceeded tumor cells disintegrated, and the proportion of these macrophages in tumor deposits increased both in lymphoid tissues and elsewhere (figs. 1a, 7–10, 13). Diagnosis of the histologic type of lymphoma increasingly became impossible in affected deposits; sometimes virtually no identifiable tumor cells remained. At the stage where macrophages predominated in a given lymphoma deposit, a fibrotic reaction began (figs. 9, 10). In advanced lesions, the macrophages were totally or partially replaced by fibrous scar tissue, in which no identifiable tumor cells remained, but in which extramedullary granulocytopoiesis occasionally appeared (figs. 14, 14a). This eosinophilic scar tissue could be extremely striking in histologic sections of the thymus and lymph nodes, where it completely effaced normal architecture (figs. 1–6, 11–13), and in the periportal areas of the liver (figs. 7–10, 14, 15), where fibrous

tissue surrounding the blood vessels and bile ducts did not normally occur in the mouse. As these changes progressed, special stains revealed increasing amounts of extracellular polysaccharides, reticulin fibers, and collagen fibers deposited in the lesions where fibrosis was developing. The entire process could be summarized as a transformation from an undifferentiated, florid lymphoma to chronic inflammation, terminating ultimately in scar formation.

The sequence of histologic changes during regression was similar in all tissues, but a given stage of regression was not attained simultaneously in every tissue. Neighboring deposits, as in adjacent periportal areas in the liver or in different lymph nodes, might in the same animal show quite different stages of regression, occasionally ranging from florid lymphoma in some to fibrosis and scar formation in others. Consequently the site of the most advanced lesion, in mice that died before complete regression, varied from one mouse to another. In some mice the site of the most advanced regression was found in the thymus, in others in the spleen and lymph nodes, and in others in the perivascular deposits in the viscera, chiefly the liver. This lack of uniformity in different lesions in the same mouse provided the means for histologic classification of the pre-existing malignant lymphoma in the large proportion of mice in which some tumor deposits remained identifiable at the time of death.

Concurrently with the regression and fibrosis of deposits of malignant lymphoma, hematopoietic tissue became atrophic. The marrow (previously invaded by lymphoma cells) became aplastic, the hematocrit dropped, and compensatory myeloid metaplasia did not develop to a significant extent. The marrow sometimes retained small foci of granulocytopenia, and the red pulp of the spleen often contained megakaryocytes and a greatly reduced amount of granulocytopenic tissue; but the liver, which should have been the site of extensive myeloid metaplasia under these conditions, rarely, if ever, developed histologically identifiable foci of extramedullary erythrocytopenia. Occasional megakaryocytes and foci of granulocytopenia were visible, the latter both in unaffected liver tissue and in regression scars, but the only evidence of red blood cell production consisted in the relatively large number of anuclear, but pyroninophilic, erythrocytes visible in the hepatic blood vessels.

It has been demonstrated (3) that the usual infectious microorganisms (*Eperythrozoon*, *Hemobartonella*), which often cause severe hemolytic anemia in mice subjected to drastic experimental procedures, played no role in the regression-aplastic anemia syndrome. No single species of microorganism was consistently isolated from the tissues of diseased mice on culture media which included selective media for the isolation of diphtheroid and acid-fast organisms, which might have acted as adjuvants to enhance immune reactivity at the site of tumor deposits. Nor were such organisms demonstrated in the tissues of such animals with methenamine silver or acid-fast staining procedures.

### Classification of Extent of Regression in Individual Mice With Lymphomas

The histologic features of malignant lymphomas in GP and NZW mice at death covered a broad and continuous spectrum from massive, florid lymphoma to complete scar formation in all affected tissues. For ease of discussion and classification, all mice were grouped into one of the four categories defined below:

1) Florid lymphoma: There was no sign of significant regression in any tissue section examined.

2) Incipient regression: Most low-power fields ( $\times 100$ ) were diagnostic of florid lymphoma, but at least 2 fields from 1 or more tissues showed massive numbers of "starry-sky" macrophages intermingled with sparse populations of disintegrating neoplastic cells or early stages of fibrosis.

3) Partial regression: Some low-power fields in 1 or more tissues were compatible with florid lymphoma, but a substantial proportion of the tissues examined contained fibrous scars in areas where lymphoma deposits would be expected.

4) Total regression: No low-power field was compatible with florid malignant lymphoma, and scar formation was advanced in all tissues examined.

Transplantation to neonatal recipients of lymphoid cells from mice in any of the first 3 categories uniformly induced lymphoid tumors in the recipients. Equal numbers ( $\sim 10^6$ ) of lymphoid cells from "totally regressed" GP mice frequently failed to establish a lymphoma transplant which correlated with the total absence of identifiable tumor cells in histologic sections from many such animals (3). Some neoplastic cells were still identifiable

histologically in tissues of most NZW mice in this category, and transplantation was successful with cell suspensions from these animals. Transplanted lymphomas did not regress, even those established from the florid stage of autochthonous neoplasms which subsequently totally regressed in the primary host (3).

### Factors Affecting Incidence of Regression

From the preceding discussion and the data presented in table 1, it is evident that the genetic background of different strains of mice is of major importance in determining the frequency and extent of the regression phenomenon. In addition to the strains listed in table 1, strains C3HfB/HeN, DBA/2N, and C57BL/6N mice have developed smaller numbers of ENU-induced lymphomas but had no sign of even the incipient stage of regression. The exact way in which genetic background determines the frequency and the extent of regression is unknown.

I have seen the regression phenomenon only in lymphomas induced by ENU, but I have not examined many mice in which lymphomas have been induced by other compounds of different chemical classes, such as the polynuclear aromatic hydrocarbon, or by fractionated whole-body X irradiation. Other investigators have indicated that they may have seen this phenomenon but did not recognize it at the time of observation in their laboratories. It must be emphasized that regression will probably not be seen in laboratories where animals are killed as soon as overt physical signs of lymphoma are observed, since regression may require a further period of several days or weeks.

### Prognosis in Mice With Lymphoma Regression

Survival time consequent to the onset of symptoms for lymphocytic or undifferentiated malignant lymphoma varied from a few days to several weeks, depending largely on whether a thymic tumor was present. Sometimes a thymic tumor would develop so rapidly that an animal with no signs of respiratory difficulty on one day would die with a large mediastinal mass on the next day. GP mice frequently developed the progressive anemia characteristic of lymphoma regression without having first exhibited unequivocal physical signs of florid

malignant lymphoma; the nature of their disease became fully apparent only following histologic examination after death. Consequently, survival times for animals which underwent regression versus those which did not were difficult, if not impossible, to compare accurately. In general, however, the life expectancy of most animals which underwent regression was not strikingly greater than that of animals which did not.

Because malignant lymphoma may develop to quite an advanced stage without causing physical signs of disease, however, the question remains: Do any animals actually survive the condition? Furthermore, since the most obviously debilitating feature of the regression phenomenon is the aplastic anemia and its sequelae, does a modified form of regression develop in which the anemia either does not occur or is reversible? Unequivocal evidence is limited, but the following observations in ENU-treated NZW/BlN mice suggest that this is indeed so.

One NZW male, given 0.75  $\mu$ mole/g ENU at 5 weeks of age, developed physical signs of respiratory difficulty identical to those seen in mice with thymic lymphoma after a latency period of 20 weeks. The condition of this mouse stabilized, however, and did not progress to the generalized lymphadenopathy and hepatosplenomegaly characteristic of disseminated malignant lymphoma. In addition, this mouse did not become anemic. Killed 6 months later at 1 year of age, it had a small, partially fibrotic, thymic tumor with limited infiltration of the mediastinal membranes by poorly differentiated lymphoma cells. It had normal hematocrit and bone marrow and no indication of lymphoma deposits or of scar formation in the viscera. The 6-month survival of this animal with this condition was most unusual and suggested that this mouse reacted to its disease in such a way as to contain its spread.

An NZW female, given 1  $\mu$ mole/g ENU at 5 weeks of age, never developed the signs of either malignant lymphoma or of anemia. It was killed and autopsied at 1 year of age when the experiment was terminated.<sup>3</sup> Histologic sections of the liver re-

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<sup>3</sup> It had several neoplasms of epithelial origin, including 10 pulmonary tumors, 2 hepatomas, and a papillary squamous polyp of the forestomach and an adenomatous polyp of the small intestine, both with malignant change.



vealed the characteristic periportal scars, of considerable magnitude and in many locations, which are features of fully regressed malignant lymphoma (fig. 15); although the kidneys were normal, the renal capsules were fibrotic and greatly thickened. Since I have not seen similar scar formation in other conditions in this species and have not seen it described by others, possibly this animal may have had a subclinical malignant lymphoma from which it apparently recovered completely. However, in other species, including the rat, the pig, and various subhuman primates, in which periportal connective tissue is normally present and even abundant in healthy animals, this observation would have no significance. It is only because of the absence of such tissue in the livers of normal mice of comparable age that this observation appears significant.

## DISCUSSION

No exact equivalent of the murine lymphoma-regression syndrome has been described, either in other experimental animals or in man. However, there are conditions in the mouse, the rat, and in man which are relevant to a discussion of the murine lymphoma-regression syndrome.

The erythroblastic phase of Friend virus infection may spontaneously regress when one specific strain of the virus is used to initiate disease in either Swiss or DBA mice (5, 6). This has been attributed to an immune reaction of these animals to this virus-induced lymphoma. Neither aplastic anemia nor a fibrotic reaction has been reported to accompany this reaction. The spontaneously occurring reticulum cell neoplasms, type B, which develop in high incidence in mice of the inbred strain SJL/J, frequently regress spontaneously on transplantation to syngeneic recipients (7). Autochthonous neoplasms do not regress, but sometimes they develop a fibrotic reaction (8). This pleomorphic neoplasm is the closest murine equivalent to Hodgkin's disease in man, in which fibrosis is often prominent (*see below*). This system has been carefully studied for the possible role of immune processes in the course of both autochthonous and transplanted tumor. Although immunity seems to play a major role in both cases—antilymphocyte serum reduces the latency period for development of these neoplasms in primary hosts (9), and the transplanted

tumors can be shown to be immunogenic (7)—it does not seem to account for all aspects of the behavior of the transplanted tumors. Other factors, possibly hormonal, also may play a role (7). In a recent experiment, it was reported that chemically induced erythroblastic leukemia in the rat was caused to regress, either in the autochthonous host or in syngeneic transplants, by hypophysectomy—perhaps the most extreme form of hormonal manipulation (10). This experiment was performed because hypophysectomy causes erythrocytic hypoplasia in the rat. Since hormones of the adrenal cortex profoundly affect lymphoid tissue, possibly manipulation of adrenocortical hormone activity may shed light on the nature of the regression phenomenon.

At least 3 conditions in man resemble in part the regression phenomenon we have observed in certain strains of mice. The fibrotic scarring which occurs in advanced lymphomas of mice most closely resembles the nodular sclerosing variant of Hodgkin's disease in man. This form occurs more frequently in women, who experience a lower incidence of Hodgkin's disease than do men and who are therefore presumed to be more resistant to its development. Also, this form is associated with a favorable prognosis (11), as is the nodular sclerotic variant of human lymphosarcoma (12). Thus, the fibrotic reaction does indicate a defense mechanism against the neoplastic process.

The human neoplasm histologically most closely resembling the florid phase of murine lymphomas which subsequently undergo regression is Burkitt's lymphoma, which is classified as an undifferentiated or poorly differentiated lymphocytic neoplasm characterized by an abundance of "starry-sky" macrophages within the tumor deposits (4). The peculiar anatomical predilections of Burkitt's lymphoma do not resemble the murine counterpart, and fibrotic reactions do not occur. But Burkitt's lymphoma often remains localized for a long time and is extremely sensitive to chemotherapeutic agents, which give long-lasting remissions and very probably cures. It has been suggested that this response to chemotherapy, so much more favorable than that of acute leukemia in childhood, results from the existence of auxiliary host-defense mechanisms against this type of neoplasm. Lymphomas histologically similar to Burkitt's lymphoma, complete with "starry-sky" macrophages, also develop

spontaneously in the dog (13) and cat (14), are likewise sensitive to chemotherapeutic agents, and have given rise to similar speculations concerning a possible immune response against these tumors (13).

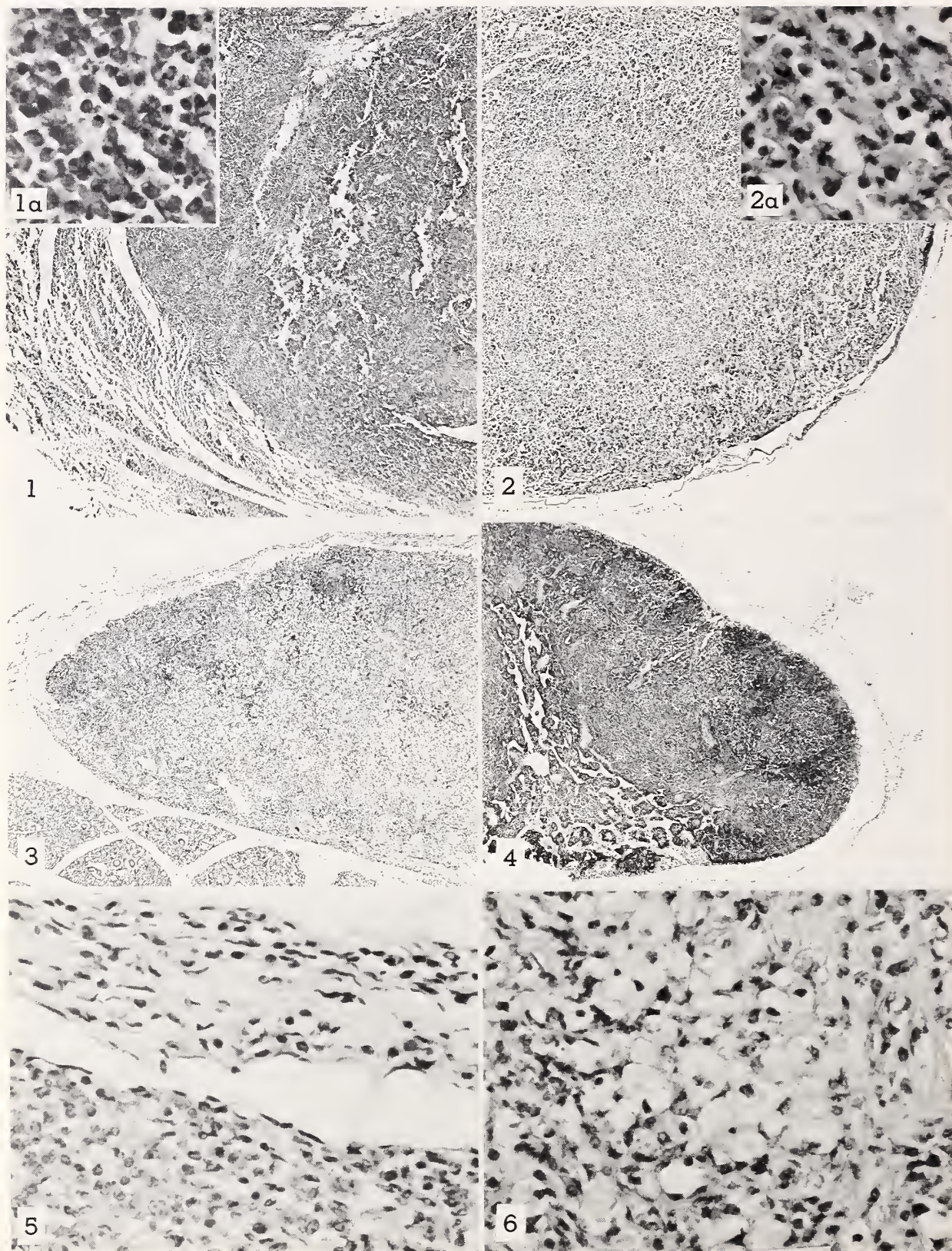
Finally, aplastic anemia sometimes precedes the development of acute leukemia in man (15). In view of the reversed sequence of these conditions in the murine lymphoma regression syndrome, one wonders whether some cases of idiopathic aplastic anemia in man might not be due to a host response against a subclinical leukemia, in which the leukemic proliferation eventually overwhelms the host response.

A major question to be investigated concerning the regression of murine lymphomas is the exact role of different components of the lymphoreticular system, both in the regression phenomenon itself and in the associated aplastic anemia, with the eventual goal of being able to direct and to control the phenomenon.

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**Figures 1-6: Lymph nodes from GP mice in various stages of lymphoma regression. All sections were stained with hematoxylin and eosin.**

FIGURE 1.—Inguinal lymph node removed surgically during florid stage. All internal structure is obliterated, and neoplastic lymphocytes extend beyond capsule of enlarged node.  $\times 40$ . *a*) Higher magnification showing dense masses of lymphocytes and macrophage (*center*) containing phagocytosed nuclear debris.  $\times 400$

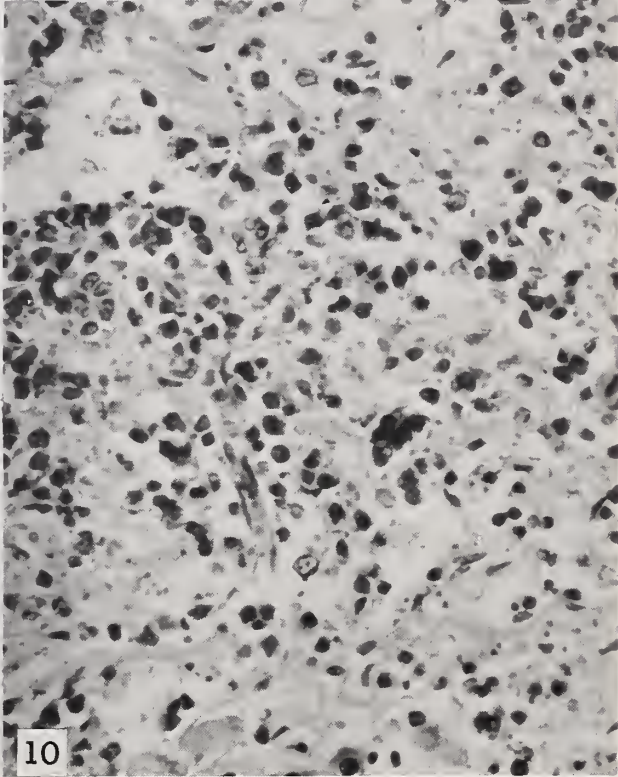
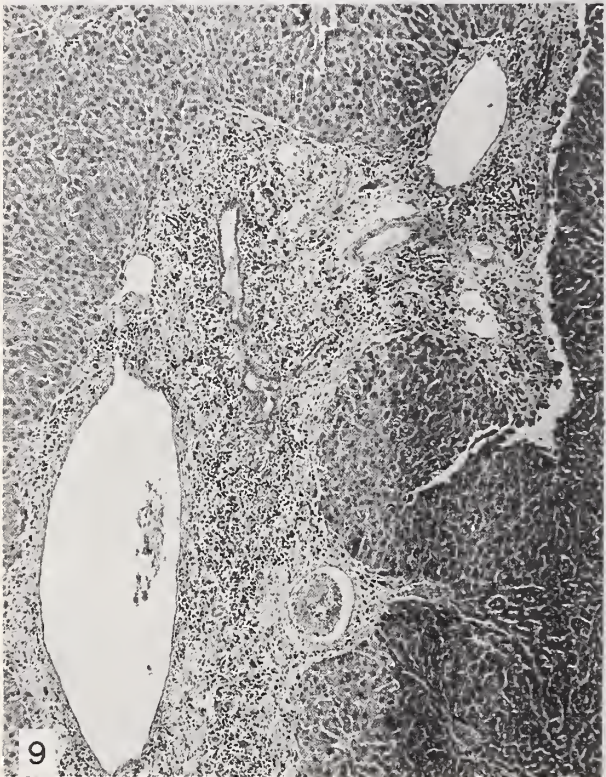
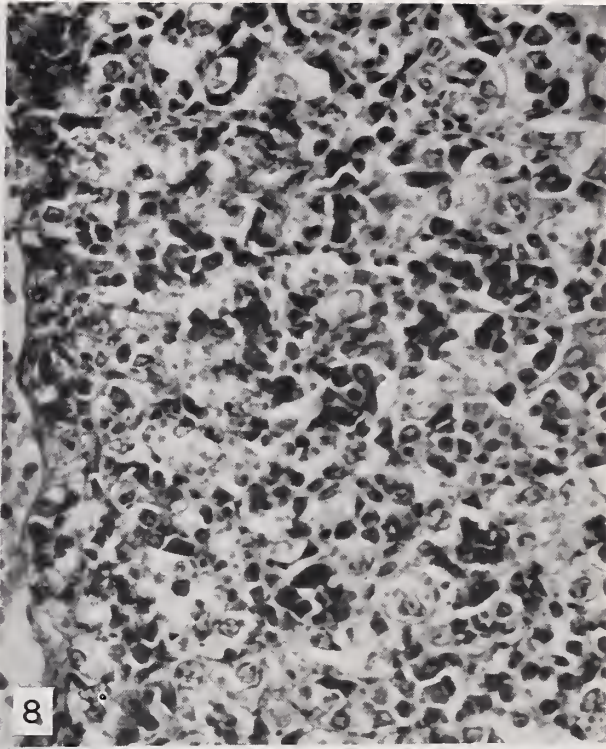
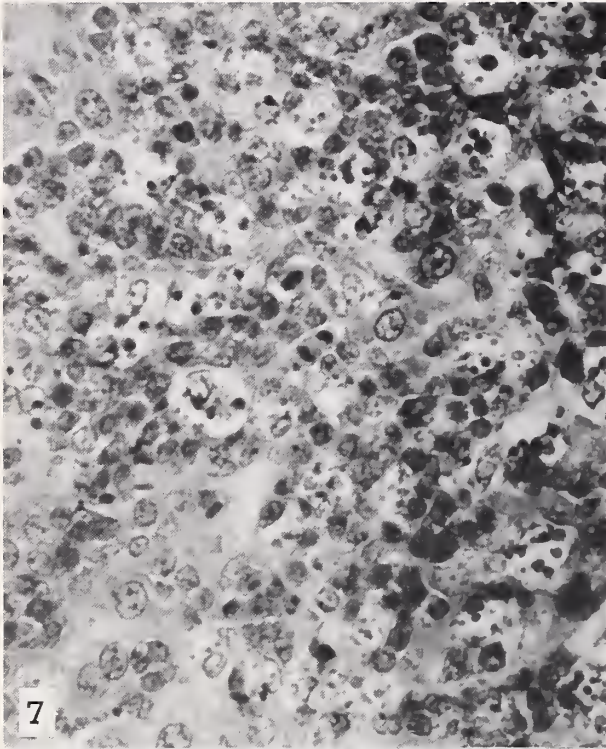
FIGURE 2.—Contralateral node removed from the same mouse 16 days later showing incipient regression. Both nodes embedded and sectioned in the same paraffin block. Neoplastic lymphocytes are less densely packed and no longer found outside the capsule.  $\times 40$ . *a*) Higher magnification of area of regressing node in which fibrosis has developed.  $\times 400$

FIGURE 3.—Cervical lymph node from a case of complete regression. Subcapsular and medullary sinuses visible, but normal architecture is effaced by fibrous tissue in some areas and by masses of large histiocytes elsewhere. No area of the node is diagnostic of malignant lymphoma.  $\times 40$

FIGURE 4.—Normal lymph node from untreated mouse.  $\times 40$

FIGURE 5.—Higher magnification of fully regressed lymph node (fig. 3) showing thickened, fibrous capsule and exaggerated subcapsular sinus.  $\times 440$

FIGURE 6.—Medulla of the same node consists chiefly of large histiocytes.  $\times 440$



**Figures 7-10: Stages of lymphoma regression as seen in liver of GP mice.**

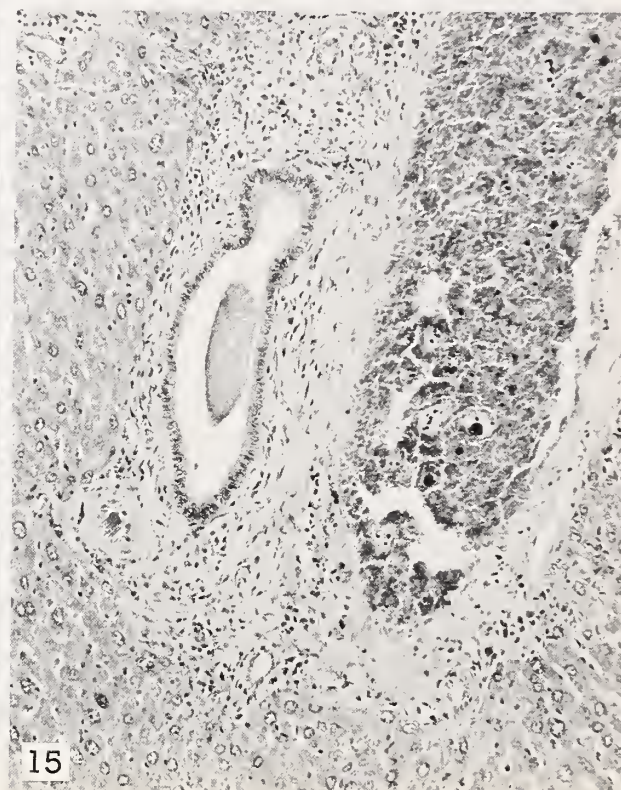
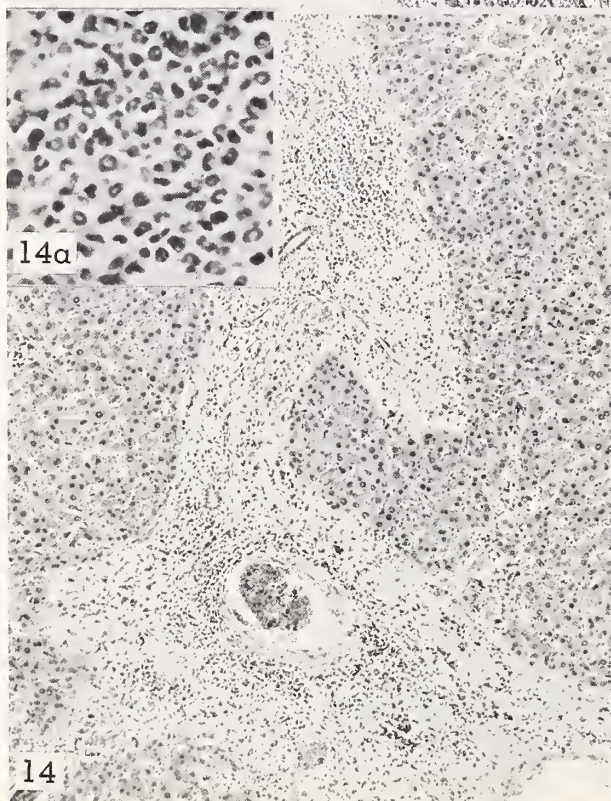
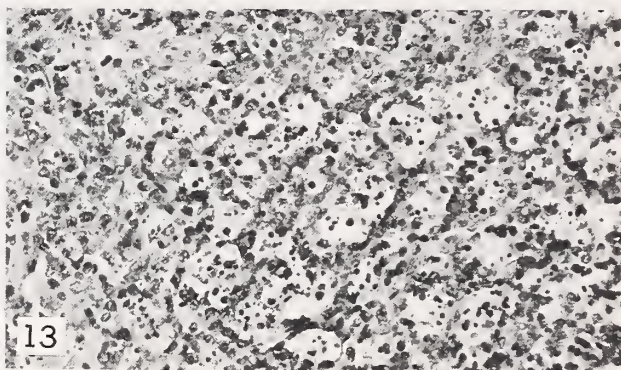
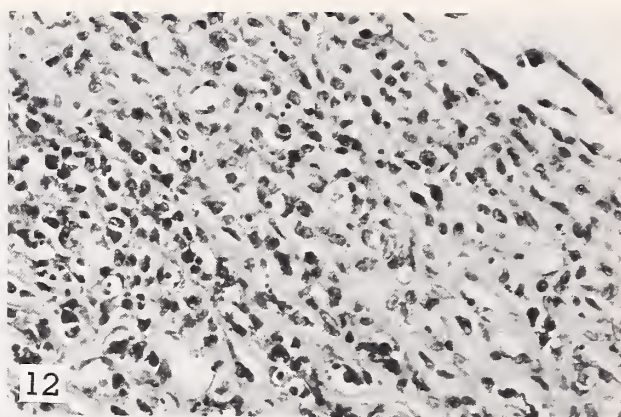
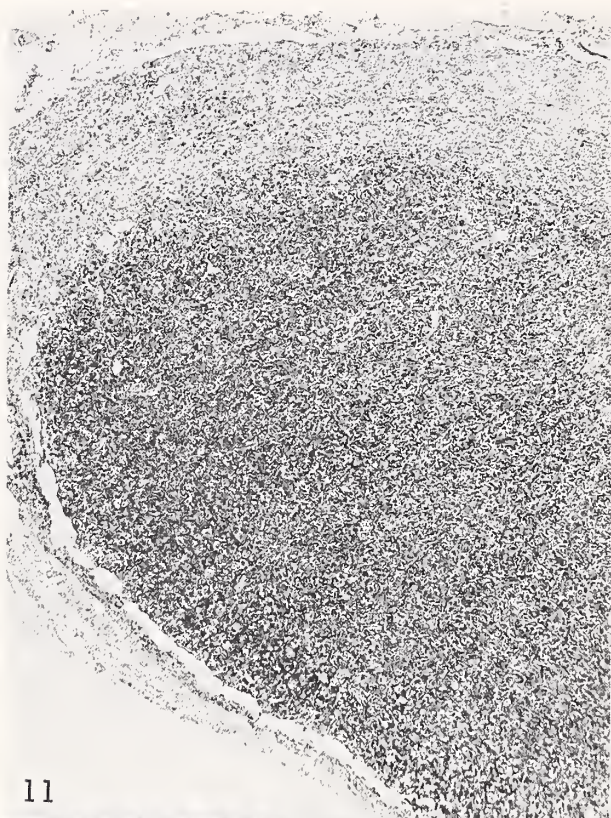
FIGURE 7.—Initial phase of regression marked by influx of unusual numbers of large, “starry-sky” macrophages into periportal tumor deposits. At this stage, neoplastic tumor cells still divide (*center*), and lesion can be unequivocally identified as a lymphoma deposit.  $\times 440$

FIGURE 8.—As macrophages become more numerous, the pleomorphic cell population (incipient regression) becomes increasingly difficult to identify as lymphoma.  $\times 440$

FIGURE 9.—In cases of partial or complete regression, the periportal lesion is an eosinophilic fibrous scar.  $\times 63$

FIGURE 10.—Residual cell population in this lesion consists of macrophages and scattered lymphoid cells; fibrosis apparent in *upper third* of this higher magnification of figure 9.  $\times 440$





**Figures 11-15: Lymphoma regression in NZW,B1N mice.**

FIGURE 11.—Lymph node from male mouse shows fibrous pericapsular tissue surrounding more darkly stained area in which lymphoma cells are still numerous.  $\times 40$

FIGURE 12.—Higher magnification of fibrous area at *top* in figure 11.  $\times 250$

FIGURE 13.—Higher magnification of residual nodule of lymphoma tissue at *lower right* in figure 11, showing abundant macrophages.  $\times 250$

FIGURE 14.—Periportal scar in liver of female mouse after complete regression.  $\times 100$ . *a*) Residual cell population within this scar consists of a focus of myeloid metaplasia.  $\times 400$

FIGURE 15.—Periportal scar in liver of female mouse given  $1 \mu\text{mole/g}$  ENU at 5 weeks of age and killed 1 year later. This mouse had never shown physical signs of either florid malignant lymphoma or of anemia. Multiple, periportal scars and thickened, fibrous renal capsules suggested that this animal may have had a subclinical malignant lymphoma from which it recovered.  $\times 160$





## Summary: Modification of Immunity and Carcinogenesis<sup>1</sup>

Nechama Haran-Ghera, *Department of Chemical Immunology, The Weizmann Institute of Science, Rehovoth, Israel*

THE SPEAKERS in Session 4 stressed the role of host immunity in tumor development and regression. It was indicated that immune impairment might contribute to tumorigenesis, nonspecific modulators of the immune response can reduce or prevent tumor development by stimulating the immunologic function of the host, and regression of a carcinogen-induced lymphocytic lymphoma may occur due to immune destruction of the tumor cells.

The immunosuppressive properties of many chemical carcinogens and tumorigenic viruses have been suggested as contributing to tumor development. Dr. Szakal pointed out the immunosuppressive properties of 7,12-dimethylbenz[*a*]anthracene (DMBA), *i.e.*, impaired humoral immunity being established using sheep red blood cells (SRBC) as the test antigen and cellular immunity impairment assessed by the homograft reaction. Studies concerned with the findings of an immunosuppressive effect caused by oncongenic viruses involved similar test methods.

At this point I would like to raise several questions: What test assays should be used when one estimates the type and degree of immune impairment, caused by one of the above agents, in relation to tumorigenesis? Many chemical carcinogens and leukemogenic viruses were analyzed in relation to their immune impairment effect by the evaluation of the response of animals to SRBC (7-8). This assay might be misleading when one tries to establish the existence of immune defectiveness in relation to tumorigenesis. Instead, assays reflecting the status of mediated immunity, considered to be involved in neoplastic transformation and/or proliferation, should be used in such evaluations.

Does the transient immune impairment of the host caused by chemical carcinogens or oncogenic

viruses actually contribute to tumor development, and would the correction of this defect change the tumor incidence? Or do we perhaps have two parallel phenomena that coincide with timing without interacting?

I would like to mention at this point some of the results we obtained concerning the induction of lymphatic leukemia and the development of spontaneous reticulum cell neoplasms in mice—experimental systems in which we could not find a direct correlation between modification of immunity caused by a leukemogenic virus, DMBA, or age increase and carcinogenesis.

In accordance with the immunosuppressive effect of different leukemogenic viruses (mainly estimated by the study of the depressed immune response of the host to SRBC), the radiation leukemia virus was found to exert a similar influence. Does this immune impairment actually contribute to lymphatic leukemia development or affect the latent period of tumor development? This problem could be tested in the following way: Mice were infected with a passage preparation of a radiation leukemia virus (inducing in 85-100% adult C57BL mice lymphatic leukemia at an average latent period of 120 days when injected directly into the thymus lobe); 30 days after virus inoculation, the spleens of these mice were removed for the evaluation of plaque formation (for each mouse) after immunization with SRBC. The splenectomized mice could be kept alive for further follow-up of leukemia development. The level of plaque-forming capacity did not correlate with leukemia development (9). Leukemia appeared in mice exhibiting either a *low* or *high* response to SRBC, and the latency was also found to be *unrelated* to the degree of immunosuppression induced by the radiation leukemia virus. The radiation leukemia virus did not significantly depress cell-mediated immunity when tested for prolongation of skin allograft survival, allogeneic tumor takes, and graft-versus-host reactivity.

A relationship between immunosuppression in-

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<sup>1</sup> Presented at Conference on Immunology of Carcinogenesis, held by the Oak Ridge National Laboratory at Gatlinburg, Tenn., May 8-11, 1972.

duced by carcinogenic agents and its contribution to tumorigenesis can be concluded only if the degree of induced transient immune impairment, by one of these agents, is estimated for *each* animal and the degree of impairment is correlated with the actual positive tumor development of each tested animal, or shortening of the latent period is evaluated in relation to the degree of host immune defectiveness.

The degree of transient immunosuppression induced by DMBA did not correlate with the incidence or latency of lymphatic leukemia development in the SJL/J strain of mice. These mice are highly susceptible to lymphatic leukemia induction—70–90% intact and thymectomized adult mice treated with DMBA develop lymphatic leukemia at an average latent period of 170 days. Studies carried out in our laboratory have shown that the leukemic cells originate in B-lymphocytes, in contrast to the virus-induced lymphatic leukemia that is dependent on the availability of T-cells. DMBA treatment in SJL/J mice exerts an immunosuppressive effect, manifested both in humoral and cell-mediated immunity. No correlation was found between the level of plaque-forming capacity to SRBC (evaluated for each numbered mouse by removal of the spleen of the mouse which was then kept alive for follow-up of leukemia development) and leukemia incidence or length of the latent period. Tests concerned with the possible contribution of impaired cell-mediated immunity induced by DMBA and leukemia incidence, estimated by prolongation of skin allograft survival for each DMBA-treated mouse (kept alive until leukemia development), showed only a *partial* correlation between cell-mediated impairment and leukemia development in relation to incidence and length of latent period of tumor development. It seems, therefore, that, although DMBA causes transient immune impairment, it most likely does not directly contribute to leukemia development.

These results are in accord with one of the conclusions mentioned previously in this meeting by Dr. Lappé, *i.e.*, that immunosurveillance commonly fails when there is carcinogen- or virus-induced immune depression.

What about spontaneous tumor development? Do the tumors occur only in aged mice when their immune response decreases? Our studies concerning the development of spontaneous reticulum cell tumors in SJL/J mice (up to 90% developing these

tumors at a mean latent period of 380 days) showed that the defective immunologic status of SJL/J mice early in life (when compared to that of other strains of mice) did not seem to play a role in spontaneous tumor development. The evaluation of the immunologic reactivity of normal and spontaneous tumor-bearing SJL/J mice with age increase (assaying the response to SRBC, the ability to develop a skin reaction of the delayed-type hypersensitivity, and the ability of lymphoid cells to cause a graft-versus-host reaction and prolongation of skin allograft survival) indicated a decrease in the immune reactivity of mice from the age of 7 months onward, irrespective of whether the host was normal or tumor bearing (10). It was interesting to demonstrate that the immune reactivity of 20 mice bearing spontaneous tumors (tested individually) at the age of 5 months was identical to that of normal mice at the same age.

At what stage in tumor development does the immune reactivity of the host contribute to tumor development? Is host immunity important for the actual neoplastic transformation or, rather, for further tumor cell proliferation?

Immunologic surveillance, if effective, is probably acting mainly at the early stages of tumor cell transformation and/or proliferation. As mentioned previously, immunosurveillance commonly fails when immune impairment is induced by chemical carcinogens or by oncogenic viruses.

Assuming that tumor cells induced by carcinogenic agents acquire new antigens not found in their normal counterpart, these new antigens are capable of inducing a host immune response. This immune response could lead to a buildup of immunization in the host that carries some tumor cells: Such a condition could prevent the proliferation of transformed tumor cells within a certain latent period. Ultimately, the breakdown of immunity and/or a cumulative increase of enhancing antibodies may finally prevail and enable tumor development after a long latent period. These events occur in C57BL mice inoculated when newborn with the radiation leukemia virus and actually developing lymphatic leukemia 5–7 months later (11).

I would like to conclude by proposing that in tumor induction systems, by chemical or viral carcinogenic agents, the immune modifications critical for the ultimate tumor occurrence are not in the early stages of neoplastic transformation, but rather in the later stages of tumor cell proliferation.

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## GENERAL DISCUSSION

**J. Stjernswärd:** Maybe we could divide the discussion into 3 areas of concern, just as Dr. Haran-Ghera suggested: 1) to ascertain whether the concept of immunologic surveillance is mature to explore for therapeutic manipulation after analysis of whether it is a reality, 2) to keep in mind the dichotomy of the immune response, and 3) in a practical given example, to analyze whether time is mature—and it may be realistic—to adapt experience from an animal model system to a specific clinical situation. I would suggest use of the model of irradiation-induced lymphopenia, in which we have started documenting effects on T- and B-lymphoid status and in which we know that a given percentage of distant metastases will later occur. A good start is the analysis done by Dr. Weiss. Is Dr. Weiss ready to apply his findings to a practical situation, or should we further analyze the various experimental systems? There is a great need for our experimental conclusions and findings to be adapted as a reality to the cancer patient. With this group of investigators, we ought to be able to reach constructive conclusions.

**G. Mathé:** You have given a good argument to show us that we don't have a good test to appreciate immunity working against and/or for cancer. This is true in animal work and in clinical work. With all the battery of tests available, we can only anticipate death in 6 months in Hodgkin's disease. Pathologists do much better to foresee prognosis in this disease than immunologists.

**Y. Maruyama:** I suppose I am the only radiotherapist in this audience. At least I'm stimulated to mount, as it were, a little response to your comments regarding radiotherapy, particularly on breast cancer.

Breast cancer is a notoriously perverse system, and, as you mentioned, practically any kind of data that you want can be obtained from the existing literature. I think you are very selective in your choice!

A lot of factors have entered into, let us say, the use of radiotherapy in this disease. Some of these, for example, derive from the work of the Manchester Clinic, where radiation was delivered to segments of the chest wall or to the peripheral lymphatics of the breast after mastectomy.

The evidence from that experience indicates that, in the irradiated area, the incidence of recurrent active disease sites was reduced from something like 1 in 5, or 6, to about 5 in a 100, indicating that disease is indeed inactivated in those sites.

That experience has been pretty adequately confirmed around the world, but one must sharply distinguish survival from local occurrence. Some of the data you used involved survival, and I think that nobody has really been able to affect the disease greatly by the use of the present therapy. We don't understand it.

A number of pictures were shown of local recurrence. Not documenting completely the dose used, the type of radiation used, the energy of the electron beam, whether cobalt was used, or the voltage is a very important consideration. The energy of the electron beam can pass through the superficial layers of the skin, leaving viable tumor in the

superficial layers. Using cobalt beams without bolus, which would bring the dose up to skin, can lead to recurrences in the skin.

Their radiated skin is not normal skin; it's dark. If you performed a biopsy around those areas, you would find recurrences outside the areas as well as within. The manifestations in those local sites may simply be the fact that the degree of resistance is altered in those particular sites.

The lymphocyte number, the absolute lymphocyte count, I would have some reservations in regarding as primary for prediction. In Hodgkin's disease, after radiotherapy, the lymphocyte counts are down. This does not alter the patients' survival or recurrence rates. In fact, after 5 years of being free of recurrences, the patients have survival curves that match those of comparably aged populations.

At least in radiation therapy of cell models of cancer of the cervix, we have also found that we can certainly get resistance to heavily irradiated tumor cells—in suppressed or unsuppressed animals. One should be somewhat cautious in describing the modality of therapy without a fuller understanding of all the aspects of it.

**Stjernswärd:** The controversy regarding the therapeutic value of postoperative irradiation in patients with early mammary carcinoma is old and not yet settled. Mortality and survival are definitely not changed, according to all available studies. Besides economic and personal negative effects, postoperative irradiation may have direct, biologically negative effects, although well-documented data demonstrate that it decreases local skin and lymph node metastases (Jackson 1964, Fletcher 1970, Easson 1968). It can certainly be discussed whether radiotherapy should be offered to these patients at a later time. The problem with many defenders of postoperative radiotherapy is "not what they don't know, but that they know so much that ain't so."

All of the 3 strictly randomized trials so far available in Europe (see my Introduction for detailed data) demonstrate an increased occurrence of, or death from, distant metastases during the first year after treatment in the postoperatively irradiated groups compared to the group that had only surgery. In all, the difference is statistically significant. Just as great as the logic is most probably for preoperative irradiation in certain forms of mammary carcinoma, just as convinced am I that the logic for routine postoperative irradiation in early mammary carcinoma is similar to "closing the stable door after the horse has bolted."

Many of these patients have—as documented by later distant metastases—"minimal residual tumors" not clinically manifested at irradiation. The radiation-induced T-lymphocyte lymphopenia may be biologically significant for the earlier occurrence of distant metastases.

However, I did not intend to discuss radiotherapy, but I wanted to make the point that the long-lasting lymphopenia and change in proportion of circulating B- and T-lymphocytes we found offer a unique opportunity for testing the degree and importance of postulated host immunity in humans and for studying manipulation of host-immune status, *e.g.*, by bacille Calmette-Guérin (BCG). To

stimulate a discussion on this and to offer a concrete model for constructive suggestions, I brought up this point.

The lymphopenia observed after postoperative radiotherapy is of the same magnitude as that seen after extracorporal irradiation—a procedure which sometimes prolongs survival of weakly antigenic transplants.

**Maruyama:** Isn't the important question, not the number, but the quality of the function that one observes?

**Stjernswärd:** My objection is that all data show clearly that postoperative irradiation does not increase survival. Do we agree on that? We should use both society's and the individual's time better and more critically.

**W. D. Terry:** I wanted to go back and pick up Dr. Boone's presentation and then bring it back to the question that you raised about immunosurveillance. The specific instances brought up in Dr. Krueger's work all relate to lymphoma and lymphoreticular neoplasms. One question we have to ask ourselves is whether the tumors developing after immunosuppression result from suppressed activity of the immune system, or whether the immunosuppressive agents really act as carcinogens or cocarcinogens. If the increased incidence of tumor is due to immunosuppression alone, why don't we see increased frequencies of the other, more common types of malignancy, such as colon or breast?

Now a comment about immunosurveillance. The concept of immunosurveillance has been an immensely useful one. It has been very productive and has generated not only heat but also a fair amount of light!

Questions now arise whether this concept has outlived its usefulness and whether we should stop attempting to either prove or disprove the existence of immunosurveillance of tumors. What is important is the definite evidence that we can utilize the immune system in the prevention or the therapy of tumors. We can immunize against viral antigens and prevent induction, and we can immunize against tumor-specific antigens and prevent induction. We can therefore utilize the immune system in immunoprophylaxis.

We also know that, in a number of different systems, we can utilize the immune system in a therapeutic way, albeit in an exceedingly limited manner at the present time.

Knowing that we can utilize the system for both prophylaxis and for therapy, need we really continue to concern ourselves with the existence of immunosurveillance, and perhaps might we not more profitably move on to other ways of looking at the relationship between host and tumor?

**C. E. Mawas:** Dr. Weiss, do you have a sequence study, in mice receiving a methanol extraction residue (MER) at different doses, of the number of thymus and spleen cells and the proportion of theta-positive cells and B-cells after such a treatment? Did you monitor also the phagocytosis ability of macrophage after such treatment? If so, is there a correlation with the observed protective or enhancing effect? Also, with which tests are you monitoring the immune status of your leukemia patient in which you are injecting MER?

**D. W. Weiss:** With regard to the question of B- and T-cells, Dr. Diane Yashpke together with Miss Abraham in our laboratory is just finishing a fairly large study on this question. All the experiments aren't completed yet,

but at this point the one fact that is quite clear is that at the levels of antigen-reactive cells, both thymus- and bone-marrow-derived cells are strongly stimulated by treatment of the animals with MER. It does appear that, with limitingly small quantities of MER, where there is no longer any effect on B-cells, there may still be a strong effect on T-cells, but I don't want to commit myself on this as yet; there are still experiments in progress.

**C. Boone:** I think I can answer Dr. Terry's question for Dr. Krueger, but first I'll rephrase it: Is the mechanism of the increased incidence of lymphoma after immunosuppression due to destruction of immunosurveillance, or is it due to increased stem cell proliferation concurrent with the immunosuppression? I know Dr. Krueger well enough to say that he is a lumpner rather than a splitter; so he would say it's due to both!

**T. Mariani:** I have 2 questions for Dr. Szakal. First I would like to ask: Are you sure that the 7,12-dimethylbenz[*a*]anthracene (DMBA)-treated skin that you are transplanting does not have malignant cells in it—just a few cells? I ask this because I will be presenting extensive evidence that, if skin taken from a tumor-bearing animal with a Gross passage A lymphoma is transplanted on a normal syngeneic host, the skin will be rejected. But the skin is rejected because of the influence of malignant cells in the transplanted skin. And, furthermore, I will point out that the rejection of tumor-bearing skin is not immunologically based.

The second question I would like to ask you is: Perhaps I've missed a very salient point in your rationale, but I fail to understand how you can say that a DMBA-treated mouse is immunosuppressed, and yet you tell us that it rejects an autograft.

**A. K. Szakal:** I cannot directly answer your first question. It is possible that malignant cells present in the skin by 11 DMBA treatments before papilloma appearance and the DMBA induces suppression of cell-mediated immunity, at which time the first autografts are rejected. On the basis of histology alone, however, I cannot make such a conclusion.

By the time the 19-times DMBA-pretreated isografts are rejected, antigenically distinct, benign papilloma cells are in the skin graft of immunologically competent recipients. The same is true for the corresponding, similarly pretreated autografts. These autografts are, however, on recipients with a depressed cell-mediated immunity resulting from the increased DMBA treatment.

To answer your second question, I believe that rejection of 19-times DMBA-pretreated isografts results from increased immunogenicity in the grafted skin, as I showed in my presentation. In the autograft pretreated the same way, rejection is due to the increased immune reactivity against the tumor, resulting from an interaction between the increased immunogenicity and the available cell-mediated immunity. This is supported by the increased papilloma regressions at that time, on the autograft, before its rejection.

**J. H. Wallace:** In view of the very obvious importance of enhancement in connection with MER administration, Dr. Weiss, would you elaborate on the enhancement phenomenon as you see it with MER administration?



**Weiss:** With regard to many tumors, pretreatment with MER either doesn't do anything or provides resistance.

With regard to some tumors, all 3 possibilities are there: enhanced resistance, enhancement which can be transferred by serum, or no effect—depending on dosage and parameters.

The one generalization I can make is that, even in those systems where MER pretreatment enhances tumor growth, if one adds to the treatment specific immunization (or in our studies, radiotherapy or chemotherapy, which may be equivalent to specific immunization by liberating tumor-associated antigens), then enhancement never seems to occur. I don't know why.

Looking at it on the cellular level in humans, I find an increase of reactive lymphoid cells, T-cells apparently (although I'm not sure this is so in all cases), but reactive attacker cells and a decrease in 7S antibody with enhancing activity. Beyond that I really can't go at this point.

**J. W. Thomas:** I would like to raise the matter of the absence of Hodgkin's disease in these immunosuppressed patients who, we are always saying, have such a high incidence of lymphomas. I'm also interested in hearing some other people's comments on the congenitally immunosuppressed patients.

In regard to reticulum cell sarcoma, it seems a peculiar cell to come up as the proliferating cell—a cell about which

we know almost nothing. Certainly few seem to be willing to place it in the role of immunology at the present time.

Another thing is that, if you say that it has to do with repeated, continuous infections, how do you explain that the overwhelming majority of lymphoma patients who come to you do not have this history? They may have a recent history of some infections, but they certainly don't have a history like the long-standing, congenitally immunosuppressed group.

**S. Deodhar:** With respect to the same comment, and the one before that, in patients with transplantation, there is a high incidence of malignancy, I just want to point out that the experience at different centers is highly variable. For instance, at Cleveland Clinic, we have performed 330 transplants in the last 8 years. I've reviewed all the autopsy and surgical pathology with respect to these; we found only 1 case of malignancy, a reticulum cell sarcoma developing in the post-transplant period. That was a very interesting case because it was one in which the reticulum cell sarcoma developed right at the site of antilymphocyte globulin injection, and if you want to make any kind of cause-and-effect relationship it was very tempting from that standpoint.

**Stjernswärd:** The figure would still stand, then, that it is about 37 out of the 3,000 getting tumors. It does not change that overall figure, so it would be very important to analyze why these differences exist between various centers. These are constructive facts to analyze.



SESSION 5

Interaction of Humoral and Cellular  
Mechanisms in Tumor Immunity

Chairmen: Karl Erik Hellström and Barry R. Bloom





# Introduction: Interaction of Humoral and Cellular Mechanisms in Tumor Immunity<sup>1</sup>

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I will discuss "Interaction of Humoral and Cellular Mechanisms in Tumor Immunity." Since several reviews have been written on this topic and also by ourselves (*i.e.*, K. E. Hellström and I. Hellström, *Adv Cancer Res* 12:167–223, 1969; *Ann Rev Microbiol* 24:373–398, 1970; and *Ann Rev Med* 23:19–38, 1972), there is no need for me to give an elaborate introduction or to express my opinions again. Therefore, I will only stress a few points and will refer the readers of this Introduction to the above reviews for details and references.

First, it is now rather well established that most tumors in animals and in man have tumor-associated (and sometimes tumor-specific) antigens, against which an immunity can be detected *in vitro* and, sometimes, *in vivo*. Most (but probably not all) of these tumor-associated antigens can stimulate the host immune response so that both cell-mediated and humoral immune reactions to the antigens develop. The immunologic response to tumor antigens is reminiscent of the response to weak histocompatibility antigens and, like responses to normal alloantigens, is largely mediated by immunologically competent cells (particularly lymphocytes but also macrophages). If one interferes with the development of the cell-mediated immune reactions, *e.g.*, by performing neonatal thymectomy or by treating individuals with immunosuppressive drugs, the frequency of primary tumors is often increased; thus, it is well known that human patients who have been immunosuppressed so as to be able to accept foreign grafts have an increased frequency of primary tumors, such as leukemias, reticulum cell sarcomas, and carcinomas. Humoral antibodies to tumor antigens may have different effects, depending on the type of antibodies, their concentration, and the tumors; *e.g.*, cytotoxic, cytophilic, "arming," and blocking antibodies exist. Most definitely, one cannot simplistically conclude that all humoral antibodies to cancer antigens are bad. It would be naive to expect that individuals whose antibody-forming capacity is damaged would necessarily fight tumors better, since their ability to destroy neoplastic cells by various means may be equally much impaired.

Second, various techniques are now available by which one can measure different aspects of both cell-mediated and humoral immune reactions to tumor antigens. Some of these techniques demonstrate killing of tumor cells upon exposure to sensitized lymphocytes, inhibition of macrophage migration upon exposure to specific tumor antigen, stimulation of DNA synthesis in lymphocytes brought into contact with specific tumor antigen, destruction of neoplastic cells by cytotoxic antibody and complement, and abrogation of cell-mediated immune reactions by blocking serum factors. More nonspecific parameters of the immune response, such as the ability to mount a delayed-hypersensitivity reaction, can also be measured both *in vivo* and *in vitro*.

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Third, by using these techniques, we can gain some understanding of cell-mediated and humoral immune responses to tumors and how they interact, though we should realize that much remains to be learned. A few aspects of the immune defense against cancer may need some extra comments. It has become evident that animals, as well as human patients, with cancer have circulating lymphocytes that can destroy cultivated neoplastic cells of the respective type, carried (or removed) from the lymphocyte donor. The degree of cell-mediated immunity to tumor antigens varies between different individuals; titrations of the minimum lymphocyte dose needed to produce significant cytotoxicity have clearly shown that patients with large tumor loads have less reactive lymphocytes than patients with small amounts of tumor (I. Hellström and K. E. Hellström, Fed Proc, 1972). The most extensive study showing depressed specific tumor immunity in patients with growing tumor is probably a recent one from Dr. Perlmann's group (to be reported on in this session). It is also evident that, although one can detect a depressed specific tumor immunity in patients with growing neoplasms, by studying the effects of relatively small ratios between lymphocytes and target cells, one can, in almost all such patients, find an immunity by working with large ratios. One major difference between the tumor-bearing and the tumor-free groups is the presence of blocking serum factors in the tumor-bearing group. There are many indications that such factors are very important. The blocking factors are probably most often complexes between antigens released by the tumor and antibodies formed by the host in response to the antigen released, but under some conditions both antigen and probably also antibody can block alone. The blocking of cell-mediated tumor immunity is often not absolute, and so, "concomitant immunity" can be often detected in tumor-bearing individuals. Animals and patients which have recovered from tumors often have so-called unblocking antibodies, which can counteract the blocking effect.

It may be a long way before tumor immunology can help us to prevent human malignancies (if ever it will), but quite likely it will within the foreseeable future lead to improved therapy and diagnosis. The evidence we will hear today for various immune responses to tumor antigens, which can be measured *in vitro* and which show some (often quite good) correlations with a host defense to tumors *in vivo*, may give us hope that what we study may one day have practical usefulness, in addition to the intellectual enjoyment it may give. Of course, everyone realizes that the *in vivo* situation may be far more complex than what we find today by studying just a few parameters and primarily using *in vitro* techniques, but this should encourage, not discourage, further work in the area.



# Lymphocyte-Mediated Tumor Cell Destruction *In Vitro*. Some General Principles and Reactivities in Human Urinary Bladder Carcinoma<sup>1,2</sup>

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**SUMMARY**—Cell-mediated cytotoxicity *in vitro* may in certain instances require thymus-derived lymphocytes. Humoral antibodies have been shown to block this cytotoxicity. However, humoral antibodies can also induce cytotoxicity of thymus-independent lymphoid cells with affinity for antigen-antibody complexes, and these 2 different antibody activities are not mutually exclusive. Knowledge of the relative importance of these mechanisms in human cancer is fragmentary. Patients with urinary bladder carcinoma have tumor-specific cytotoxic lymphocytes and humoral antibodies. Lymphocyte cytotoxicity can be inhibited with tumor-specific glycoproteins. The cytotoxicity of the lymphocytes seems to be partially independent of specifically sensitized T-cells, but the composition of the circulating effector cell populations may differ in various phases of the disease. In contrast to what has been described for other human tumors, the specific cytotoxicity *in vitro* of the patient's blood lymphocytes is well correlated to the clinical course. Lymphocyte cytotoxicity reflects the presence of a locally growing tumor or of tumor-derived material, but is not detectable when tumor growth is extensive or metastatic.—*Natl Cancer Inst Monogr* 35: 223-229, 1972.

A VARIETY of new techniques has demonstrated tumor-associated immune responses in practically every human tumor examined (1-3). These responses are of both the humoral and the cellular type. Cellular anti-tumor responses have primarily been studied by measurement of lymphocyte-mediated tumor cell destruction *in vitro*. The notable result in this area is the apparent tumor specificity of the reactions. The effector cells, usually lymphocytes from the patient's blood, destroy tumor cells of relevant histogenetic origin in both autochthonous and allogeneic combinations but do not seem

to attack normal cells from the same tissues or cells from tumors of unrelated origin (2).

This paper deals with lymphocytic effector mechanisms of tumor cell destruction, as revealed by these common *in vitro* procedures. The first part briefly summarizes some important facts which recently have emerged from studies, in many laboratories, on different cytotoxicity models. The second part discusses some implications in regard to tumor-associated immune responses in human urinary bladder carcinoma.

## DIFFERENT CYTOTOXICITY MODELS

Table 1 lists various mechanisms of lymphocytic cytotoxicity *in vitro*. It is well established that lymphocytes from sensitized donors destroy cells that carry the relevant antigen. This reaction has been studied in a large variety of immune situations in

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TABLE 1.—Cytotoxicity of lymphocytes

1. a) Lymphocytes from donor sensitized to target cell antigen.  
b) Lymphocytes sensitized *in vitro* to target cell antigen.
2. Lymphocytes from normal donor + humoral antibodies to target cell antigen.
3. Lymphocytes activated by stimulant [phytohemagglutinin (PHA), purified protein derivative (PPD), etc.], unrelated to target cell antigen.

both animals and man (4). More recently, it has also been shown that specific sensitization of lymphocytes can be achieved *in vitro*, e.g., in a mixed lymphocyte culture reaction (5) or by exposure of normal lymphocytes to foreign transplantation antigens on fibroblasts (6).

The second model (table 1) implies that humoral antibodies to antigens on the surface of target cells can induce cytotoxicity of lymphoid cells from non-immune donors, as well as that of "nonsensitized" lymphocytes in an immune donor. Although this reaction has primarily been studied in heteroimmune situations (4), there is good evidence that it also occurs in transplantation immunity (7), in autoimmunity (8), and in tumor systems (9).

The third model (table 1) indicates that activation of lymphocytes by mitogens, such as PHA, or by antigens unrelated to target cell antigens also may induce a cytotoxic response. When induced by PHA, this reaction reflects a reactivity of thymus-derived lymphocytes (4). This model, primarily studied to elucidate mechanisms of lymphocytic cytotoxicity, will not be discussed here.

Table 2 lists some additional facts on the reactions of the first model. These facts are based on work with lymphocytes from inbred mice in immune reactions comprising H-2 alloantigens. These reactions have been shown to require thymus-derived lymphocytes, and T-cells seem at least in certain situations to be the only necessary effector cells. Thus, treatment of effector cells with alloantibodies for the T-cell-specific  $\theta$ -alloantigen abolishes the reaction (10). In addition, medullary thymus cells, transferred into irradiated allogeneic hosts, so-called "educated thymus cells," acquire a specific cytotoxicity for the host's H-2 antigens (11, 12). Specifically, cytotoxic mouse T-cells are not retained on columns carrying antigen or anti-mouse immunoglobulin (13-15). Furthermore, T-cell cytotoxicity is not inhibited by antibodies to mouse immunoglobulin (16). However, it is blocked by

humoral antibodies to the relevant target cell antigen, and this is believed to be an *in vitro* correlate of the enhancement phenomenon (17, 18). In addition, an apparent "desensitization" can also be achieved if the effector cells are left for some time on monolayers of target cells carrying the proper antigen (15, 19).

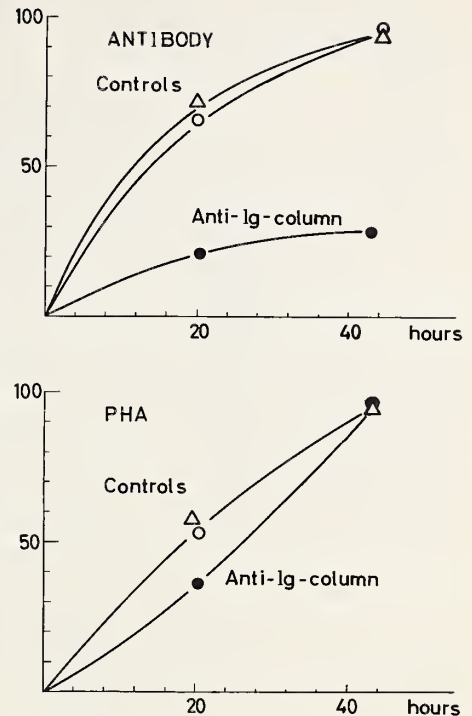
This evidence for T-cell cytotoxicity is substantial. However, these data do not allow the generalization that specific cytotoxicity of lymphocytes from an immunized donor in all situations reflects an exclusive T-cell reactivity, or any T-cell reactivity. Evidence for cooperation between sensitized T-lymphocytes and macrophages has been reported (20). Moreover, when antibodies or antibody-producing cells are present, effector functions may also be exhibited by various cell types. That cytophilic antibodies or antigen-antibody complexes may induce phagocytic and toxic reactivities in mononuclear phagocytes and polymorphs has been known for a long time.

Recent evidence indicates that certain antibodies also induce cytotoxicity in cells which, by all presently available criteria, appear like lymphocytes. While antibodies of the IgM class are inactive in this respect, IgG antibodies are highly active (21, 22). The Fc part of the antibodies is required for induction (23). Antibodies which have not reacted with antigen are not adsorbed to lymphocytes. On the other hand, antigen-antibody complexes are readily adsorbed (24). Cytotoxicity can therefore be induced in 2 ways. 1) Lymphocytes can interact with antibody-carrying target cells; this reaction requires only few molecules of antibody per target cell, and only the antibody-carrying cells are destroyed (21, 22). 2) Lymphocytes can adsorb antigen-antibody complexes and reutilize the adsorbed antibody in a cytotoxic reaction against antigenic target cells which have not seen antibody before (24). In accordance with this, lymphocytic cyto-

TABLE 2.—Cytotoxicity of mouse lymphocytes sensitized to H-2 alloantigens

1. Anti- $\theta$  antibodies combined with complement abrogate cytotoxicity.
2. "Educated" thymus cells are cytotoxic.
3. Cytotoxic cells are not retained on columns charged with anti-mouse immunoglobulin.
4. Cytotoxicity is not inhibited by anti-mouse immunoglobulin serum.
5. Cytotoxicity is inhibited by antibodies to relevant target cell antigens.
6. Cytotoxic cells are adsorbed on target cell monolayers.

TEXT-FIGURE 1.—Cytotoxicity of highly purified human blood lymphocytes to chicken erythrocytes labeled with  $^{51}\text{Cr}$ . *Upper diagram*: cytotoxicity induced by rabbit anti-chicken erythrocyte serum (final dilution  $10^{-5}$ ). *Lower diagram*: cytotoxicity induced by PHA (Burrows Wellcome, London, England; final concentration  $2\ \mu\text{g}$ ). *Ordinates*: percent  $^{51}\text{Cr}$  released from cells to medium after incubation times shown on *abscissas*. Lymphocyte : erythrocyte ratios 6.25 : 1. *Solid circles*: lymphocytes passed through column charged with rabbit anti-human immunoglobulin. *Open circles*: lymphocytes passed through columns charged with human immunoglobulin. Columns prepared as described in (14). *Open triangles*: lymphocytes passed through nylon column (24).



toxicity is easily inhibited by antigen-antibody complexes in which the antibodies are directed against third party antigens (25). Target cell lysis is by a nonphagocytic but contact- and energy-requiring process in the complete absence of complement (22). Lymphocyte activation is reflected by increased uropod formation, but blast transformation is not necessary (4, 26).

There is good evidence that antibody-induced cytotoxicity of lymphoid cells does not require participation of T-lymphocytes (table 3). Thus, removal of T-cells, either by anti- $\theta$  treatment (27) or thoracic-duct drainage (28), increases the cytotoxicity of purified lymphocyte preparations. On the other hand, the reaction is easily inhibited by treatment of the effector cells with antibody to lymphocytic immunoglobulin light chains present on the surface of B-cells (29). The reactive cells are also easily removed when the effector cells are poured through columns loaded with antigen-antibody complexes or with anti-immunoglobulin antibodies. Text-figure 1 illustrates this with the results of an experiment in which the cytotoxicity of highly purified human blood lymphocytes for  $^{51}\text{chromium}$  ( $^{51}\text{Cr}$ )-labeled chicken erythrocytes was measured after passage of the effector cells through glass bead columns, loaded with either rabbit anti-human

IgG or human IgG. This procedure, which efficiently removes B-cells (14), strongly reduced antibody-induced cytotoxicity. On the other hand, it hardly affected the PHA-induced cytotoxicity which represents a T-cell reaction.

From this brief summary it is obvious that lymphocytic cytotoxicity *in vitro* can be exhibited by cells which differ widely in their functional requirements and, most importantly, in the way in which they interact with humoral antibody. The relative function of these different mechanisms *in vivo*, for example, in a tumor situation, is largely unknown. However, clearly any attempt to elucidate the possible role of immune surveillance for tumor growth *in vivo* requires further analysis along these lines.

TABLE 3.—Antibody-induced cytotoxicity

1. Removal of T-cells increases cytotoxicity of lymphocyte preparation.
2. Effector cells are retained on columns charged with antigen-antibody complexes or with antibodies to lymphocytic immunoglobulin.
3. Effector cells are inhibited by antigen-antibody complexes (third party antigen) or by antibodies to lymphocytic immunoglobulin.



## IN VITRO STUDIES OF HUMAN URINARY BLADDER CARCINOMA

For the last 3 years we have studied the tumor-directed immune response in human urinary bladder carcinoma. We have looked for humoral antibodies and cytotoxic lymphocytes by using the microplate test as described by Takasugi and Klein (30). In this test, target cells are allowed to attach to the bottom of the wells of microtitration plates. Lymphocytes in moderate excess, or patients' serum, or both, are then added, and cell destruction is recorded after 1–3 days. Cytotoxicity is usually expressed as reduction in number of surviving tumor cells, compared with that obtained with lymphocytes from control persons. In the initial studies, the target cells were explanted, autochthonous or allogeneic, bladder tumor cells in primary cultures. Control target cells were tumor cells of other origin, autochthonous bladder epithelial cells, allogeneic fibroblasts, epitheloid Chang liver cells, and others. The effector cells were blood leukocytes or purified blood lymphocytes from patients with bladder carcinoma. Control lymphocytes were from patients with unrelated tumors or from healthy blood donors (31, 32).

The published results may be summarized as follows. Leukocytes or lymphocytes were cytotoxic to bladder tumor cells, whereas those from the controls, including cystitis patients, were not. Within the limits of the method, there was no difference in degree of destruction of autochthonous or allogeneic bladder tumor cells. Cytotoxicity was apparently specific for bladder tumor, since control tumor cells or normal cells were not destroyed. Sometimes these controls also included autochthonous, normal bladder epithelial cells. Patients' sera also contained antibodies of seemingly similar cross-reactivity when tested in complement-dependent cytotoxicity experiments without lymphocytes. Moreover, some sera also contained antibodies, or complexes, which blocked the cytotoxic effect of lymphocytes. We also found an opsonizing IgM antibody which destroyed tumor cells in the presence of C' and nonlymphocytic blood leukocytes (32).

More recent studies have aimed at further establishing the specificity of the reaction and the nature of the lymphocytic effector cells. These studies were greatly facilitated by access to 2 established cell lines of bladder carcinoma origin (33). Table 4

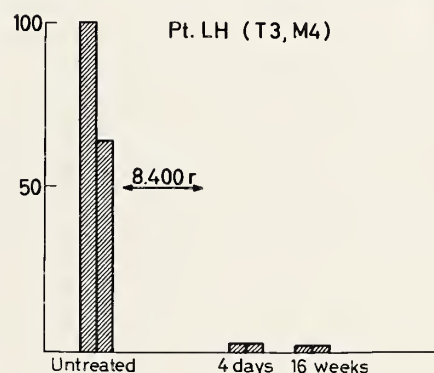
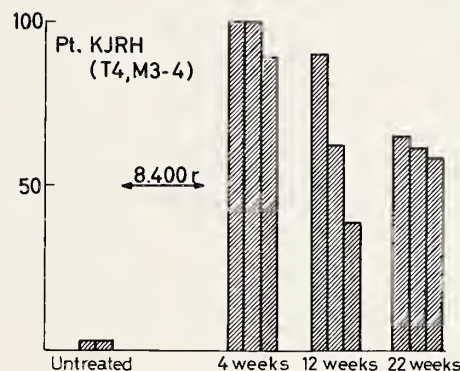
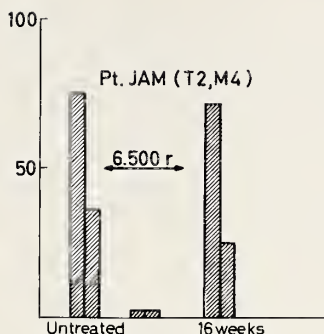
TABLE 4.—Cytotoxicity of lymphocytes from bladder tumor patient to bladder tumor cells of different origin\*

Target cells	E:T	Lymphocytes of:		Reduction (%)
		Control	Patient	
Allogeneic	500:1	97 ± 32	1 ± 1	99
primary tumor	250:1	122 ± 35	1 ± 1	99
(2d passage)	125:1	98 ± 36	11 ± 2	89
Established	500:1	77 ± 39	8 ± 10	90
cell line	250:1	113 ± 38	42 ± 32	63
(T24)	125:1	130 ± 51	81 ± 22	38

\* Lymphocytes tested at 3 different effector cell : tumor cell ratios (E:T). Cytotoxicity after 2 days' incubation given as % reduction of surviving tumor cells (No. of surviving tumor cells exposed to control lymphocytes = 100%). For details, see (33).

shows an example in which the reaction of the cells from one of these lines was compared with that of an allogeneic primary culture. The slight difference in intensity of destruction seen here was due to growth pattern of the target cells and cellular heterogeneity of the primary cultures rather than to qualitative antigenic differences (33).

Some preliminary evidence for the specificity of the reaction was obtained in blocking experiments in which soluble tumor extract was added to the incubation mixtures. The bladder tumor and control extracts used in these experiments were prepared by extraction with phenol-water; they contained glycoprotein, polysaccharides, and some nucleic acid. While most of these extracts were made from primary bladder tumors, one was prepared from a secondary bladder tumor growing in the arm muscle. When tested at concentrations ranging from 50–250 µg/ml, most of these extracts significantly reduced the cytotoxicity of bladder patients' lymphocytes when tested on cells of one of the bladder tumor cell lines. The control extracts used in these experiments were perchloric acid or phenolic extracts of colon cancer and were all high in carcinoembryonic antigen activity (34). However, none of them was active in the present test. The only exception, giving an apparent inhibition of cytotoxicity, was liver glycogen. This was assumed to reflect a nonspecific feeder effect, since this preparation enhanced survival of tumor cells in the controls as well. The blocking effect of the bladder tumor extracts was not correlated to their content of glucose. Results have so far been positive with 6 different bladder tumor extracts, including one tested in an autologous combination.



TEXT-FIGURES 2, 3.—Cytotoxicity of lymphocytes from bladder tumor patients to bladder tumor cells before and after radiotherapy. *Ordinates*: percent reduction of tumor cells after 2 days of incubation with lymphocytes (see table 4) at times given on *abscissas* (lymphocyte : tumor cell ratios are 500 : 1, 250 : 1, or 125 : 1, designated by first, second, and third column in each group, respectively). Text-figure 2: patient JAM, clinical stage T2, malignancy grade M4. Text-figure 3, *upper*: patient KJRH, clinical stage T4, malignancy grade M3-4; *lower*: patient LH, clinical stage T3, malignancy grade M4. Total doses of 6500 or 8400r of irradiation were given as described in (33).

### CORRELATION OF LYMPHOCYTE-MEDIATED CYTOTOXICITY *IN VITRO* WITH CLINICAL COURSE OF THE DISEASE AND TREATMENT

While these studies established that a tumor-associated cellular response occurs in this disease, they did not cover the relationship of this reactivity to surveillance of tumor growth in the patients. This problem was approached in an extensive double-blind study in which cellular reactivity was investigated in relation to clinical tumor staging and therapeutic treatment.

Of the patients included in this study, 19 had

stage T2 tumors (infiltration of superficial muscle), 18 stage T3 (palpable, infiltration of deep muscle), and 15 stage T4 (tumor fixed to pelvic wall or invading adjacent organs). Control lymphocytes were either from matched patients with unrelated tumors who had undergone similar treatments or from healthy controls. The treatment in this series was exclusively radiotherapy, in which high doses (totally 4500-8400 rads) were given locally according to 2 slightly different schedules (33).

The results published in detail elsewhere (33) can be summarized as follows. Before irradiation, almost all patients with tumors of stage T2 had cy-

TABLE 5.—Cytotoxicity in relation to tumor growth during 1st year after radiotherapy\*

Cytotoxicity	Residual tumor	Distant metastases	Tumor free
Strong	0	0	11
Weak	5	1	0
None	2	4	0

\* For details, see (33).

totoxic lymphocytes (14/16). In contrast, less than half of those with tumors of stage T3 and T4 had cytotoxic lymphocytes (4/9 and 3/8, respectively). During radiotherapy, reactivity disappeared completely in practically all patients. However, cytotoxicity often reappeared quickly after cessation of therapy. This was seen in all patients with tumors of stage T2 and almost all patients with tumors of stage T3 (5/5 and 8/10, respectively). In contrast, the postirradiation response of patients with tumors of stage T4 was more variable (4/8). Text-figures 2 and 3 show some examples.

On closer analysis, it was obvious that postirradiation cytotoxicity was well correlated to clinical course of the disease (table 5). Thus, while all patients who were clinically tumor free during the 1st year after radiotherapy also had strongly cytotoxic lymphocytes, all of those whose lymphocytes were inactive either developed distant metastases or had large residual tumors within a few months. Moreover, all of those whose lymphocytes after irradiation were only weakly cytotoxic also developed residual tumors or distant metastases within 1 year. These data suggest that the assay of lymphocyte cytotoxicity is of considerable prognostic value.

The question as to how long this postirradiation cytotoxicity lasts was examined in an additional group of 13 patients, whose lymphocyte cytotoxicity was assayed from 1–10 years after radiotherapy. Most of those who were clinically tumor free had no cytotoxicity. This suggests that, in the long run, lymphocyte cytotoxicity reflects the presence of a growing tumor or of some tumor-associated material (33). This conclusion was also supported by the study of another 10 patients, most of them having stage T2 tumors and treated only by local tumor resection or total cystectomy (35). In 8 of these patients cytotoxicity disappeared rapidly when they were clinically tumor free, whereas 2 with recurrent tumors also had cytotoxic lymphocytes. In contrast, in patients locally irradiated before surgery, cytotoxicity was seen for several months in the absence of a tumor.

## CONCLUSIONS

Thus in bladder carcinoma, lymphocyte cytotoxicity varies with the course of the disease. Strong cytotoxicity after radiotherapy seems to be advantageous. On the other hand, absence of cytotoxicity may reflect 2 fundamentally different clinical conditions. First, it may indicate that the patients have at least temporarily been cured of their tumors. In this case, lack of cytotoxicity means, most certainly, absence or strong reduction in number of circulating effector cells. Second, it may reflect extensive tumor growth, metastases, and a bad prognosis. Whether lack of cytotoxicity in the latter cases represents blocking or desensitization phenomena, or has some other causes, is presently not known. For full appreciation of the significance of these findings, variations in cellular reactivities must be correlated with those in humoral antibodies. Furthermore, since thymus-dependent and thymus-independent effector cells interact with humoral antibodies or antigen-antibody complexes in a completely different manner, the nature of the effector cells in the different clinical situations must also be studied.

We have recently approached the latter problems by fractionating the patients' lymphocytes by passage through columns loaded with rabbit antibodies to human immunoglobulin (14). In some cases, passage through such columns reduced the cytotoxicity of the patients' lymphocytes, thus indicating that part or all of this reactivity was due to thymus-independent effector cells. However, in other cases, column passage did not seem to decrease cytotoxicity, suggesting that it reflected a T-cell reactivity (15). Until we know more about the nature of the effector cells in different phases of the disease, it seems premature to speculate on the significance of variations in their *in vitro* reactivities for surveillance of tumor growth in the patients.

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## Relationship Between Blocking and Unblocking Activity and Tumor Growth *In Vivo*<sup>1, 2</sup>

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**SUMMARY**—Evidence indicates that tumor growth is facilitated *in vivo* by factors which are present in tumor-bearing individuals. These serum factors can specifically block tumor-cell destruction *in vitro* by lymphocytes immune to the tumor-associated antigens in question. Counteraction of the blocking activity *in vivo* inhibited tumor growth. Unblocking procedures are thus of potential interest as a new approach to immunotherapy.—*Natl Cancer Inst Monogr* 35: 231–235, 1972.

LYMPH NODE cells and circulating lymphocytes from tumor-bearing animals and human patients are specifically cytotoxic to cultured tumor cells from the same individuals *in vitro* (1–6). Sera of tumor-bearing individuals contain factors, possibly tumor antigen-antibody complexes (7), that can specifically block tumor-cell destruction *in vitro* by lymphocytes immune to the tumor-associated antigens in question (8–11). Also, some tumor antisera have an “unblocking” effect; *i.e.*, when added *in vitro* to sera from tumor-bearing animals they cancel out the blocking activity of the latter sera (12, 13). A similar activity was demonstrated in a number of human patients from whom tumors had been extirpated (14).

These findings raise the following questions: Are the blocking factors partially responsible for the progressive tumor growth in individuals having cytotoxic lymphocytes and, if so, can tumor growth *in vivo* be inhibited by inoculation of unblocking sera?

We present the available evidence indicating

that the blocking factors are of importance *in vivo* by facilitating tumor growth and that procedures which counteract the blocking activity *in vivo* are of potential interest as a new approach to immunotherapy.

### Correlation Between Presence of Serum-Blocking Activity and Tumor Growth

Factors that can specifically block the cytotoxic effect of immune lymphocytes are regularly demonstrable in the serum of various experimental animals and human patients when they carry a tumor (8, 10). The appearance of this blocking activity during development of primary and grafted experimental neoplasms has been studied. Blocking occurred more than 1 week before a tumor mass was palpable in most rats which developed primary neoplasms after polyoma virus infection as newborns (5, 15). In this tumor system, the titer of the blocking activity increased rapidly and reached its maximum level when the tumor was detectable (15). It stayed at this maximum level until the rats died. After rats were given tumor isografts, the blocking activity appeared in the serum of most animals 1 or 2 days before a tumor mass was palpable. Also in these animals, the titer increased rapidly, reached its maximum soon after the tumor was first palpated, and remained at this level during the period of tumor growth.

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The blocking activity disappears rapidly after tumor excision. In the rat polyoma system, it decreased to undetectable levels as early as 4–6 days after tumor excision. Before or when tumor growth recurs, the blocking activity is again detectable in serum.

Thus the appearance of blocking activity in serum is an early rather than a late phenomenon in tumor development. This is compatible with, but by no means proves, the hypothesis that blocking factors influence tumor outgrowth.

#### **Elution of Blocking Activity at Low pH From Fresh Tumor Tissue Obtained From Experimental Animals or Human Patients**

In several tumor systems, the blocking activity can be absorbed out from an active serum by specifically antigenic, cultured tumor cells (7, 16). It can subsequently be recovered from the washed cells by elution at low pH (7). If the antigenic receptors are expressed and exposed to the blocking factors *in vivo*, one would expect the blocking factors to be adsorbed on the surface of the tumor cells. Whether this is true was studied in rat polyoma tumors and in a few different human neoplasms (17–19). Tumor tissue was cut in small pieces and washed extensively to get rid of plasma contained in the tissue. To test for the efficiency of the washing procedure, the pieces were incubated with phosphate-buffered saline for 1 hour, stirred, and centrifuged. The supernatant was then concentrated and tested for blocking activity. The tissue pieces were incubated for another hour at pH 3 to elute possible antibodies. The supernatant was adjusted to pH 7.4, dialyzed against the phosphate buffer, concentrated, and tested for blocking. While no blocking activity was released during 1-hour incubation at pH 7.4, the subsequent incubation at a low pH regularly eluted blocking activity. This blocking eluate had the same properties as those of the blocking sera obtained from tumor-bearing individuals. The blocking activity was specific for the tumor type in question, could be counteracted by admixed unblocking serum, and caused a similar enhanced growth of tumor isografts as a corresponding serum. In the rat polyoma system, it was also demonstrated that no similar activity could be eluted from normal kidney tissue of the same animals.

Low pH eluates of tissues from tumors induced by

chemicals in mice and of tissues from tumors induced by simian virus 40 in hamsters reportedly contain IgG2, but very little or no IgG1, which indicates that the tumor cells *in vivo* are coated with IgG2 antitumor antibodies (or antigen-antibody complexes) (20, 21).

In conclusion, tumor cells growing *in vivo* are often coated with antibody-associated blocking factors. The tumor antigens are expressed and exposed to the blocking factors, and an efferent type of blocking similar to that demonstrable *in vitro* seems to occur at least to some extent also *in vivo*.

#### **Facilitation (Enhancement) of Growth of Tumor Isografts by Transfer of Blocking Serum or Eluate**

There is a way that one can clarify whether the blocking activity, as detected *in vitro*, has a similar function *in vivo*—protecting tumor cells from otherwise cytotoxic lymphocytes. One can administer the blocking material at a dose sufficient to reach a detectable level of activity in the serum of the recipients and study whether the growth of tumor isografts is facilitated in such animals compared to animals similarly inoculated with appropriate control material. A recent study on the rat polyoma system used this procedure (18). Blocking serum or eluate could be administered in such a quantity that the sera of the recipients became positive for blocking for 1–3 days. Similar inoculation of eluate from normal kidneys of tumor-bearing animals, from non-cross-reacting, 3-methylcholanthrene-induced tumors, or of control sera did not produce blocking activity in the sera of the recipients. The recipients of blocking material had the same level of lymphocyte cytotoxicity as that of the controls when tested *in vitro* with graded numbers of lymphocytes.

Also, the IgG2 eluted from chemically induced mouse sarcomas reportedly enhances tumor growth *in vivo* (22). It has not been clarified, however, whether the purified IgG2 antibodies have blocking activity, as tested *in vitro*. There are also some other recent reports on tumor enhancement caused by serum of tumor-bearing animals (and therefore probably containing blocking activity) (23, 24).

#### **Counteraction of Blocking *In Vivo* and Its Effect on Tumor Growth**

Blocking may be counteracted either by the prevention of the blocking antibody formation—prob-

ably achieved to some extent by splenectomy (25)—or by interference with the action of the blocking antibody. A way to counteract already formed blocking factors was indicated by the demonstration that some antitumor sera can abolish the blocking activity of an admixed blocking serum. Such unblocking activity was first demonstrated in serum of mice in which sarcomas induced by Moloney virus had spontaneously regressed (12). It was also demonstrated in the sera of rats from which isografts of tumors induced by polyoma virus had been extirpated (13). In these rats the serum abolished the blocking activity of an admixed serum obtained from the same animal at the time it carried a tumor. Subsequently the blocking activity was also demonstrated in the sera of human patients who were "tumor free" after surgery (14). The unblocking activity is associated with serum immunoglobulins and can be specifically absorbed out by cultured tumor cells of the type in question.

Sera with unblocking activity could also be produced by immunization procedures. Rats or rabbits were primed with bacillus Calmette-Guérin, and 10–21 days later they were inoculated with syngeneic and xenogeneic cells, respectively, from a polyoma tumor. Unblocking activity appeared in the serum 2–4 days later (13, 26).

When inoculated into rats that had received an isograft of a polyoma tumor, unblocking serum inhibited the appearance of (or counteracted) the blocking activity of serum in 4 of 5 rats. Tumors in these 4 rats grew progressively for 10–12 days and then completely regressed (13).

Thus, antibodies against tumor antigens are sometimes associated with a blocking activity that inhibits the rejection of tumor cells and are sometimes associated with an unblocking activity that favors such a rejection. These dual effects indicate that different kinds of antitumor antibodies possibly but not necessarily belong to different immunoglobulin classes, each being responsible for one of the two mentioned effects. Since some evidence indicates that the blocking activity, as tested *in vitro*, is associated with antigen-antibody complexes (7), one might consider the possibility that the two types of antibodies differ mainly in their capacity to form blocking complexes. This question has not been resolved and the mechanism of unblocking remains to be elucidated.

Encouraged by the finding that the serum-blocking activity could be inhibited by inoculation of

unblocking serum, we attempted to counteract the blocking activity of rats which already carried grossly visible, primary, kidney sarcomas when the treatment was initiated (26). Tumor-bearing rats were splenectomized and inoculated intravenously or intraperitoneally every 2 or 3 days with 2- or 3-ml volumes of unblocking serum. To study the effects on tumor growth serially obtained serum samples from the animals were assayed for blocking and unblocking activity and for homologous complement-dependent cytotoxicity. The volume of tumorous kidneys was measured and the survival times were recorded. Serum-blocking activity decreased or disappeared in all rats treated with unblocking serum, whereas no such alteration was seen in control animals inoculated in parallel with control serum. While homologous, complement-dependent, cytotoxic activity was never detected in sera of untreated or control-treated rats, it was found on several occasions in all tested rats inoculated with unblocking sera. Unblocking activity was demonstrated in several of these animals.

The inoculation of unblocking serum combined with splenectomy led to disappearance of grossly visible tumor in 2 of 11 rats and to retardation of tumor growth rate in 7 of 9 others. The mean survival time of the treated rats was twice that of the controls.

Although the demonstrated inhibition of the growth of already manifest, fairly large, primary tumors may have been induced mainly by the action of the cell-mediated, antitumor immunity, when this was no longer counteracted by the blocking factors, it is likely that at least one other alternative mechanism has to be considered. Thus the unblocking sera also contained complement-dependent, cytotoxic activity. Such activity also became detectable in the sera of the recipients. The relative efficiency *in vivo* of such cytotoxic antibody activity compared to the cell-mediated cytotoxicity cannot be judged at present. These mechanisms can be further clarified if the unblocking activity can be separated from the complement-dependent cytotoxicity.

The therapeutic effect demonstrated against fairly large primary tumors, which never regressed spontaneously, is of particular interest, since the immune manipulations were not combined with any conventional treatment. To evaluate further the therapeutic potential of unblocking, techniques must be developed to produce sera with high titers,



most ideally, to induce the production of unblocking antibodies in the tumor-bearing individual himself. Furthermore, such treatment should be combined with procedures that increase the efficiency of the cell-mediated immunity and also with partially effective, nonimmunologic types of therapy.

## CONCLUSIONS

The close correlation between the presence of blocking serum activity and progressive tumor growth in a variety of tumor-host systems suggests that the blocking activity is important *in vivo*. The elution experiments show that blocking factors not only are present in the serum but also are specifically adsorbed to the surface of at least some tumor cells *in vivo*, which thus suggests some degree of an efferent type of blocking. More direct evidence for the effectiveness *in vivo* of the blocking factors is provided by the demonstration that administration of blocking serum or eluate to recipients of tumor isografts specifically facilitated tumor growth. Since the level of cell-mediated immunity was not altered by this treatment, apparently the enhanced tumor growth was the result of a central or an efferent blocking rather than of an afferent one. The serum-blocking activity can be counteracted *in vivo* by unblocking serum alone or combined with splenectomy. This leads to an inhibition of tumor growth in the rat polyoma system, thus providing further evidence for the importance of blocking factors in allowing progressive tumor growth *in vivo*. Unblocking could be used as a new approach to immunotherapy.

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## Stimulation of Lymphocytes by Autochthonous Cancer<sup>1, 2</sup>

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**SUMMARY**—Lymphoid cells from lymph nodes (LNC) draining tumors or circulating blood lymphocytes (CBL) were mixed *in vitro* with biopsy specimens of autochthonous malignant and nonmalignant cells in which DNA synthesis had been blocked by mitomycin C, in a mixed-lymphocyte-target-interaction test. After contact *in vitro* with autochthonous tumor cells, CBL from 45 of 136 (33%) patients with active cancer were stimulated to increased DNA synthesis. A serum-mediated factor which blocked the ability of the tumor cells after incubation to stimulate autochthonous lymphocytes was found in 37 of 46 autochthonous sera, in 25 of 40 allogeneic sera from donors with "related" tumors, but in only 3 of 17 and 7 of 45 sera from allogeneic donors with "unrelated" tumors and from healthy controls, respectively. Nonstimulating tumor-biopsy cells could stimulate after elution at low pH. LNC draining large tumors with a history of long duration were specifically nonreactive against autochthonous tumor cells. In the search for a valid test reflecting the cancer patient's immunity to autochthonous cancer, fresh biopsy cells were used, because, with or without antibody coating, they may be a more representative target cell than even, for example, autochthonous cells selected through tissue culture. Whether the lymphocyte stimulation test by autochthonous cancer reflects a valid *in vitro* parameter for the *in vivo* situation remains to be elucidated.—*Natl Cancer Inst Monogr* 35: 237-242, 1972.

THE EXTENT to which the lymphocytes of patients with active cancer recognize autochthonous tumor cells immunologically was investigated. Peripheral blood lymphocytes or cells from local lymph nodes were mixed *in vitro* with autochthonous

malignant and nonmalignant cells in which the DNA synthesis had been blocked.

In the search for a valid test reflecting the cancer patient's immunity toward his own tumor cells, fresh biopsy cells were used as stimulating agents. Such cells, with or without antibody coating *in vivo*, may constitute more representative target cells than ones selected, for example, through tissue culture.

Negative controls were various normal cells treated with mitomycin C. Positive controls were stimulated with allogeneic cells and phytohemagglutinin.

Our earlier studies showed that circulating blood lymphocytes (CBL) of certain, but not all, patients are stimulated to increased DNA synthesis by

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autochthonous tumor-biopsy cells treated with mitomycin C (7-10). Autochthonous sera blocked the stimulating ability of stimulating sarcoma cells (10). Immunoglobulin coating of tumor cells *in vivo* was demonstrated (11-13). Therefore, at least some nonstimulating tumors may be blocked *in vivo* by interaction with the serum (6, 10).

The present study not only analyzes the validity of the positive stimulation but also briefly analyzes the frequency with which CBL react against autochthonous cancer, and whether serum-blocking factors exist in fresh, *in vivo*, biopsy-tumor cells.

Preliminary data indicated that lymphoid cells from lymph nodes (LNC) exposed to small doses of reinjected tumor cells were stimulated by autochthonous tumor cells slightly more frequently than lymphoid cells from the contralateral non-tumor-exposed glands (4, 5). A clear difference in weight was also documented (5). Also, in the present study, the ability of CBL to be stimulated will be compared with that of LNC draining tumors of large size and long duration.

## MATERIALS AND METHODS

**Patients.**—All patients had an active neoplastic disease when their lymphocytes were tested against autochthonous tumor cells. Peripheral blood was collected before surgery.

**Lymphocyte stimulation test.**—The mixed-lymphocyte-target-interaction test (MLTI) was done as detailed in (2, 9).

TABLE 1.—Stimulation of lymphocytes by autochthonous tumors from biopsies\*

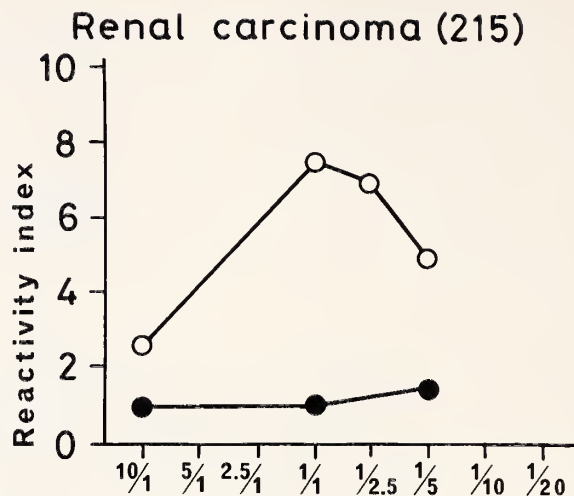
Tumors tested	Number positive/ No. tested
Brain tumors†	6/29
Sarcomas‡	20/44
Carcinomas§	14/29
Nasopharyngeal carcinomas	5/15
Myeloma	0/3
Burkitt's lymphoma	3/16

\* Only reactivity indices >2.0 and statistically significant were considered positive. They were calculated as the ratio between the isotope uptake in a test sample and the autochthonous, nonmalignant, mitomycin C-treated cells mixed with lymphocytes. All values were first corrected to the background.

† Glioma and astrocytoma.

‡ Chondrosarcomas, fibrosarcomas, liposarcomas, myxosarcomas, neurofibrosarcomas, osteosarcomas, synovial sarcomas, and reticulum cell sarcomas.

§ Renal, mammary, thyroid, and liver.



TEXT-FIGURE 1.—Dose-response kinetics. Stimulating ability of renal carcinoma cells (○—○) and nonmalignant kidney cells (●—●) on autochthonous lymphocytes. Vertical bar: reactivity index; horizontal bar: tumor cells:lymphocytes.

**Tumor cells, lymphocytes, and lymph nodes.**—They were collected and separated as described in (5, 10).

**Serum incubation.**—This was done as described in (10).

**Elution.**—This was performed as described in (14).

## RESULTS

### Ability to Stimulate CBL by Autochthonous Tumors

Previous studies showed that peripheral CBL may be stimulated to increase their DNA synthesis by exposure to fresh, mitomycin C-treated, autochthonous tumor cells from biopsies (7-10). Not all tumor cells stimulate, and their frequency to stimulate autochthonous lymphocytes may vary among the different types of malignancies. The frequency of positive stimulation in the first 136 tumors tested is summarized in table 1. Only reactivity indices >2.0 and statistically significant were considered positive. About a third of the tested tumors (45/136) stimulated the patients' CBL. This frequency may vary considerably for brain tumors, lymphoid tumors, sarcomas, or carcinomas.

The dose-response kinetics were investigated for various ratios of lymphocytes:tumor cells. Whenever possible, we included nonmalignant cells from the tissue from which the tumor originated.

TABLE 2.—Inhibition of lymphocyte stimulation by preincubation of the tumor cells\* with various sera

Serum-tumor relation	Serum from	Type of tumor	Number† showing inhibition after incubation/No. tested
Autochthonous	Autochthonous	Sarcomas	31/38
	"	Carcinomas	3/3
	"	Brain tumors	3/5
Allogeneic, "related"	Allogeneic sarcomas	Sarcomas	17/29
	Allogeneic carcinomas	Carcinomas	8/11
Allogeneic, "unrelated"	Brain tumors	Sarcomas	1/10
	Carcinomas	"	0/1
	Sarcomas	Carcinomas	0/2
	Brain tumors	"	2/4
Healthy	Healthy donor	Sarcomas	6/34
	"	Carcinomas	0/7
	"	Brain tumors	1/4

\* Effect of preincubation tested in an autochthonous lymphocyte tumor combination showing positive stimulation.

† Total number includes repeat tests of the same sera.

The antigenicity of various tumors may differ and thus, also, the optimal ratio. Different types of malignancy were, therefore, tested. The results of this test will be detailed in (75). A typical dose-response curve is shown in text-figure 1. The optimal ratio of stimulating ability of lymphocytes: tumor cells was usually 1:1. An excessively high or low proportion of tumor cells would not stimulate.

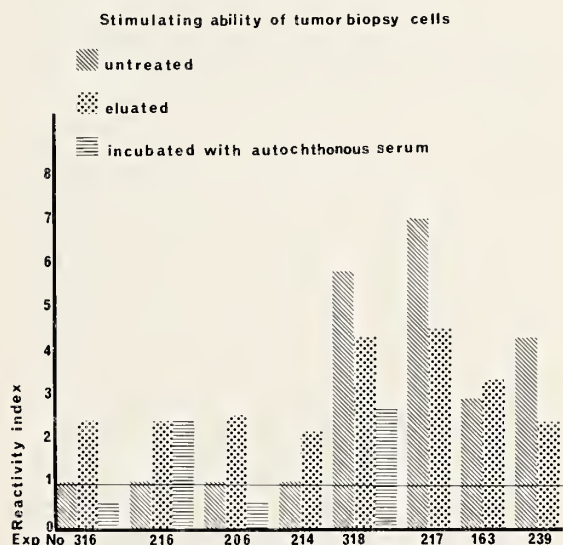
The results thus indicate that positive stimulation is not accidental. The dose-ratio curves achieved resembled the curves characteristic for stimulation of lymphocytes by other antigens, *e.g.*, bacterias or histocompatibility antigens.

#### Serum-Mediated Inhibition of Lymphocyte Stimulation by Autochthonous Tumors

The results on inhibition of lymphocyte stimulation by preincubation of the tumor cells with various sera are summarized in table 2. Significant blocking was obtained with 37 of 46 autochthonous sera in test combinations in which the lymphocytes were significantly stimulated by autochthonous tumor cells. In 25 of 40 tests, inhibition was effected by allogeneic sera from patients whose tumors were of the same histologic category as the tumor cells stimulating autochthonous lymphocytes. Sera from patients with "unrelated" tumors blocked in 3 of 17 cases, and those from healthy donors in 7 of 45 cases.

#### Lymphocyte Stimulation by Eluted Tumor Cells From Biopsies

Elution and blocking tests with the same tumor cells may be one way to detect possible antibody coating *in vivo*. When testing with the fresh biopsy cells, we never know if the cells will stimulate. Therefore, the same tumor cells were tested blindly



TEXT-FIGURE 2.—Lymphocyte stimulation by eluted tumor cells from fresh tumor biopsies as compared to stimulating ability of untreated tumor cells or eluted cells after preincubation with autochthonous serum.

TABLE 3.—Comparison of the stimulation of LNC draining large tumors and CBL by autochthonous tumor

Stimulated cells	Stimulating cells treated with mitomycin C	Number positive/ No. tested	
		Tumor stimulating CBL	Tumor not stimulating CBL
CBL	Autochthonous tumor	8/8	0/5
"	" LNC	6/7	1/5
"	Allogeneic lymphocytes	8/8	5/5
LNC	Autochthonous tumor	2/8	2/5
"	" CBL	0/8	0/4
"	Allogeneic lymphocytes	8/8	5/5

either as fresh biopsy cells or after elution at pH 7.2 or 3.1. Eluted tumor cells were also incubated with autochthonous serum and then tested for stimulating ability. Text-figure 2 demonstrates the first 8 tumors tested. Results are grouped according to whether the fresh, uneluted tumor cells stimulated. Untreated, fresh, biopsy cells from the first 4 tumors did not stimulate autochthonous lymphocytes. All 4 samples did so, however, after elution, and reactivity indices around 2 resulted. When the eluted tumor cells were incubated with autochthonous serum, 2 of 3 of these tumor cells lost their ability to stimulate. All 4 tumors whose fresh biopsy cells had stimulated (#316, 217, 163, and 239) still had cells which stimulated after elution.

#### Reactivity of Tumor-Draining Lymph Node Cells and CBL to Autochthonous Tumor

The ability of autochthonous tumor cells to stimulate LNC draining tumors of large size and long duration has been compared with that of CBL from the same patient, in the same experiment, with cells from the same tumor. The stimulating ability of CBL by autochthonous draining LNC was also tested, as was the ability of CBL to stimulate LNC. Thirteen patients were tested. The results are grouped according to whether autochthonous tumor cells stimulated CBL (8 patients) or did not stimulate CBL (5 patients). In the 8 patients in whom autochthonous tumor cells stimulated CBL, the same tumor cells did not stimulate LNC (0/8). The lack of stimulation of the LNC was not due to nonviability or nonreactivity because they were stimulated, and usually to the same degree as the CBL, by allogeneic lymphocytes. LNC draining the tumor significantly stimulated

CBL, whereas the reverse was not observed (table 3).

#### DISCUSSION

The present results, demonstrating that not every tumor stimulates autochthonous lymphocytes, seem reasonable if one considers that cancer in a patient represents a situation in which a postulated immunologic control system has failed or has been outflanked. A constant finding of positive stimulation would immediately cast doubts on the relevance of the *in vitro* results to the *in vivo* situation. The occurrence of tumor-associated immune reactions in human cancer is now fairly well established (16, 17) and does not need to be confirmed here. However, an important question has arisen: To what extent are the various *in vitro* data representative of the *in vivo* situation and not merely indicative of *in vitro* artifacts selected by the experimenter?

The validity of positive stimulation by autochthonous tumor cells as a test reflecting tumor-associated immunity is supported by 4 findings: 1) the dose-response curves, 2) the different distribution of reactivity indices when malignant and nonmalignant biopsies of the same tissue origin are compared, 3) the reproducibility of positive stimulation, and 4) the indicated tumor-associated specificity of the various sera tested for blocking ability. Whole tumor cells have also been found in other laboratories to stimulate significantly autochthonous lymphocytes (18-21).

As in many cases, however, the exception rather than the expected may be most interesting. Here, the nonstimulating tumor cells may in certain, although not all, instances reflect escape mechanisms such as enhancement.

Preincubating tumor cells with autochthonous serum may abrogate or reduce the tumor cells' ability to stimulate. Together with the results of the elution experiments, this suggests that the stimulating factor is localized at the surface of the tumor cells and that serum, most probably antibodies, can block the stimulating properties of the tumor cells. The concept of antibody coating *in vivo* is thus supported by the present results as a possibly realistic escape mechanism. The phenomenon of immunologic enhancement was demonstrated in experimental systems *in vivo* (22), and the exposure of target cells to humoral antibodies could abolish



the cytotoxic effect of cellular immunity *in vitro* (23–25). Results, parallel with ours, obtained from measurement of tumor-lymphocyte interaction were found in allogeneic lymphocyte stimulation experiments, in which preincubation with specific anti-HL-A serum abrogated the lymphocyte's stimulating ability (26).

Mixed lymphocyte culture inhibition by serum was found in transplantation experiments to be a valuable assay for detection of preformed antibody correlating with graft prolongation (27–29). Whether in our MLTI test a parallel correlation exists between tumor growth or spread and occurrence of blocking factors is open to test.

The results of the blocking experiments described indicate a certain tumor-associated specificity of the MLTI test. Through the analysis of blocking factors in serum, an immunologic parameter distinguishing various types of cancer may be established.

It is as yet unclear whether antibodies only or antigen-antibody complexes (30) cause the observed inhibition. F(ab')<sub>2</sub> fragments were suggested as another candidate (13, 28).

There are several explanations for the specific, tumor-associated, nonstimulating ability of the LNC draining the tumor. All LNC come from patients with large tumors of long duration. Specific anergy, *e.g.*, paralysis or tolerance of the tumor-responding clones, may be one explanation, but presently this explanation cannot be differentiated from the interpretation that the clone's ability to respond to the tumor is exhausted, or in other words, all cells able to respond have already been transformed. The lymphocyte stimulation test has, in fact, been interpreted as a test able to demonstrate tolerance *in vitro* (31–33).

The stimulation of CBL by LNC and not vice versa favors the interpretation of paralysis due to excess tumor products such as antigens. Lymph nodes which drain the site of antigen injection can trap a relatively constant proportion of antigens (34). Furthermore, antigens associated with macrophages are more immunogenic than the native antigen (35).

The size of the tumor and the duration of exposure to it *in vivo* may be critical factors. Thus the present findings are opposed to other findings indicating that, when tumors are small, as in early mammary carcinoma, LNC are immunologically active against the tumor cells *in vitro* (36).

Whether increased DNA synthesis of lymphocytes exposed to tumor cells reflects presensitization *in vivo* or a primary immune response *in vitro* is open to question. The phenomenon of antigenically stimulated lymphocyte transformation *in vitro* has been interpreted as predominantly reflecting the proliferative immune responses associated with *in vivo* delayed hypersensitivity and has been suggested to detect immunologically committed "memory" cells (37).

Thus at present not only immunity but also certain escape mechanisms, such as enhancement and possibly tolerance, may be documented in man with the same test. Whether our *in vitro* results are relevant to the *in vivo* situation can be elucidated only by further studies.

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# Functional Capacity of Cytotoxic Lymphoid Cells<sup>1</sup>

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**SUMMARY**—High levels of cell-mediated cytotoxicity could be demonstrated by further immunization of sensitized cells in X-irradiated hosts and subsequent *in vitro* assessment with the use of a rapid chromium-51-release assay. Under these conditions, the cytotoxic lymphoid cell, without destroying itself, could specifically destroy more than one target cell by direct contact. Direct contact lysis occurred without detectable involvement of cell-free mediators of cytotoxicity.—*Natl Cancer Inst Monogr* 35: 243–249, 1972.

CONSIDERABLE EVIDENCE indicates that tumor-bearing hosts have immune lymphoid cells which inhibit tumor cells *in vitro* (1). Why these immune cells do not eradicate tumor within the host is one of the basic questions facing tumor immunologists. In a broad sense, we can postulate that the host's immune cell response is either intrinsically insufficient for tumor rejection or is dampened by host-regulatory mechanisms. Our interest here is in the last possibility.

The present experiments illustrate high levels of cytotoxic lymphoid cell activity, which can be achieved under conditions of minimal host regulation. Highly active cytotoxic cells were produced by adoptive transfer of sensitized mouse spleen cells, together with fresh antigen, into X-irradiated syngeneic recipients (2). Donor spleen cells were sensitized against an allogeneic lymphoid leukemia. Activity of cytotoxic lymphoid cells increased 200- to 400-fold above levels found with donor cells in the first 6 days after adoptive immu-

nization (manuscript in preparation). Host-regulatory mechanisms suppressing development of cytotoxic lymphoid cells presumably are by-passed by the adoptive-transfer procedure. Cytotoxic cells were further purified by bovine serum albumin (BSA)-gradient purification. These procedures were used to obtain cytotoxic lymphoid cells whose functional capacity for tumor cell destruction *in vitro* was investigated. The average cytotoxic cell, without destroying itself, appeared able to specifically kill more than one tumor cell by direct, cell-mediated lysis. The relationship of this finding to host-tumor interactions is discussed.

## MATERIALS AND METHODS

*Animals, tumors, and immunizations.*—Adult male C57BL/6 and BALB/c mice were reared by the Animal Production Unit of the National Institutes of Health. EL 4 lymphoid-leukemia cells, originally induced in C57BL/6 mice by a chemical carcinogen (3), were used for immunizations. BALB/c hosts were given variable numbers of weekly intraperitoneal injections of  $3 \times 10^7$  EL 4 tumor cells washed in saline. LSTRA, a lymphoid leukemia induced by Moloney virus in BALB/c mice, was used for specificity controls. Both EL 4 and LSTRA are passaged as ascites tumors.

*Adoptive-transfer procedure.*—Spleen cell suspensions were prepared from BALB/c hosts which had been sensitized by 6–8 weekly injections of  $3 \times 10^7$

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EL 4 cells. Between 2 and 3 spleens were teased apart in balanced salt solution (BSS), passed through gauze to remove particulate matter, and washed by centrifugation ( $200 \times g$ ) with 40-ml iced BSS. Spleen cell suspensions were mixed with fresh, viable EL 4 tumor cells and injected intravenously into nonsensitized BALB/c hosts X-irradiated the previous day with 400 R. Optimal immune response in spleen cells assayed 6 days after the transfer procedure was obtained by injection of  $10^7$  EL 4 cells and  $10^6$  nucleated viable spleen cells in 0.25 ml BSS. A Westinghouse Quandrocondex X-ray unit (Baltimore, Md.) was used at 200 kv, 15 ma, and a half-value layer of 1 mm copper. Radiation, 140 R/minute, came from 2 tubes 54 cm apart.

**BSA-gradient cell separation.**—Discontinuous BSA gradients (4) were prepared in  $2 \times 1/2$  inch Beckman cellulose nitrate tubes (Palo Alto, Calif.), with the use of 0.8 ml each of 33, 29, 27, 25, and 23% BSA (Pentex Path-o-cyte 5; Miles Laboratories, Kankakee, Ill.) diluted in BSS. Osmolarity of stock BSA was adjusted to 290–300 milliosmoles/liter with hypertonic sodium chloride or distilled water prior to use. In 0.5 ml BSS, 10–50 million spleen cells were layered on the gradient, and tubes were spun at  $9300 \times g$  (mid-tube) for 30 minutes in a Beckman Model L ultracentrifuge with an SW 50.1 swinging-bucket rotor. Bands were removed by hand with Pasteur pipettes, and cells were washed once with 40 ml iced BSS. Cytotoxic lymphoid cell activity is concentrated by BSA-gradient separation (5); in our hands, cytotoxic cells localized predominantly at the 25 and 27% BSA bands.

**Cytotoxicity assay.**—Cell-mediated cytotoxicity was determined by a modification of the chromium-51 ( $^{51}\text{Cr}$ )-release technique introduced by Brunner, Mauel, Cerottini and Chapuis (6). The assay has been detailed elsewhere (7). Target cell damage induced by immune cells was calculated by the following formula:

$$\text{Percent lysis} = \frac{IC - NC}{FT - BG}.$$

*IC* represents target cell  $^{51}\text{Cr}$  released by immune spleen cells; *NC* represents target cell  $^{51}\text{Cr}$  released in the presence of normal, nonsensitized spleen cells; *FT* represents target cell  $^{51}\text{Cr}$  released by freezing and thawing target cells 4 times in an ethanol-dry-ice bath; and *BG* represents gamma-

counter background. Target cell incubations with immune and normal spleen cells were in quadruplicate. Standard errors of the percent-lysis values were approximated as described elsewhere (8).

Uptake of trypan-blue dye was used as an independent assay for target cell death in one set of experiments. Visual counting of cells was facilitated by BSA-gradient removal of dead cells, red cells, and subcellular debris from immune and normal lymphoid cells before the cytotoxicity assay.

## RESULTS

### Multiple Target Cell Destruction by a Single Immune Cell

Immune spleen cells from adoptively immunized hosts were enriched for cytotoxicity by BSA density-gradient purification and incubated *in vitro* with  $^{51}\text{Cr}$ -labeled target cells. Target cell damage was measured both by  $^{51}\text{Cr}$  release and uptake of trypan blue (table 1).

$^{51}\text{Cr}$  release correlated with uptake of trypan blue with variations in either incubation time or concentration of attacking cells (table 1, expts. 1 and 2). Consequently, in the present system  $^{51}\text{Cr}$  release from target cells mixed with immune cells appears to represent complete release of available  $^{51}\text{Cr}$  from target cells killed by cytotoxic lymphoid cells rather than increased metabolism of target cells or partial release of available  $^{51}\text{Cr}$  from all target cells. This conclusion agrees with findings of Sullivan *et al.* (9).

Table 1 also shows that the average immune-effector cell could destroy more than one target cell. This finding is illustrated in experiment 1, by target cell death after 240 minutes' incubation with immune cells. A total of  $5 \times 10^4$  attacking cells destroyed  $6.75 \times 10^4$  (estimated by  $^{51}\text{Cr}$  release) and  $7.45 \times 10^4$  (estimated by uptake of trypan blue) target cells. These results underestimate immune-cell cytotoxic potential, since more target cells would have been destroyed had the reaction proceeded more than 240 minutes. Moreover, it is unlikely that all of the  $5 \times 10^4$ , viable, nucleated, spleen cells were cytotoxic lymphoid cells. Additional examples of an immune cell destroying more than one target cell are provided in experiments 3 and 4 of table 1.

TABLE 1.—Comparison of  $^{51}\text{Cr}$  release and uptake of trypan blue by target cells incubated with cytotoxic lymphoid cells\*

Experiment No.	Incubation time (min)	BALB/c anti-EL 4 immune cells/ml†	EL 4 target cells/ml‡	Percent $\pm$ standard error of mean	
				Lysis§	Uptake of trypan blue
1	120	$10 \times 10^4$	$5 \times 10^5$	$16.3 \pm 0.4$ (2.5)	$20.3 \pm 3.8$ (3.6)
		$5 \times 10^4$	"	$8.1 \pm 0.3$ (2.5)	$7.9 \pm 1.5$ (4.5)
	240	$10 \times 10^4$	"	$25.4 \pm 0.2$ (4.6)	$25.3 \pm 3.7$ (8.3)
		$5 \times 10^4$	"	$13.5 \pm 0.8$ (4.6)	$14.9 \pm 3.0$ (2.0)
2	90	$10 \times 10^4$	"	$8.7 \pm 0.3$ (5.4)	$11.0 \pm 1.6$ (9.0)
		$5 \times 10^4$	"	$4.5 \pm 0.2$ (5.0)	$4.7 \pm 1.8$ (5.6)
	180	$10 \times 10^4$	"	$14.8 \pm 0.3$ (11.7)	$17.4 \pm 2.2$ (12.9)
		$5 \times 10^4$	"	$7.6 \pm 0.4$ (11.9)	$9.5 \pm 1.6$ (9.6)
3	240	$20 \times 10^4$	"	$65.0 \pm 2.8$ (4.5)	Not done
4	240	$5 \times 10^4$	"	$24.7 \pm 3.7$ (7.6)	Not done
		$25 \times 10^4$	"	$12.5 \pm 1.1$ (7.6)	Not done

\* Assay proceeded as described for  $^{51}\text{Cr}$ -release detection of target cell damage in "Materials and Methods." Incubations of immune cell-target cell mixtures and normal cell-target cell controls were stopped by transferring individual dish contents to individual test tubes, adding 1 ml of iced BSS per sample of reaction media, and pelleting cells by centrifugation at  $800 \times g$  for 10 minutes. To count cell-free  $^{51}\text{Cr}$ , 1 ml of cell-free supernatant was sampled. Remaining medium in each tube was discarded. Each cell pellet was then resuspended in 0.2 ml of iced BSS plus fetal calf serum and mixed with 0.2 ml of 0.2% trypan blue. Percent dead cells was determined by uptake of trypan blue.

† Single cell suspension was from pooled 25 + 27% BSA-gradient bands. Gradient-purified cells were from immune spleens of 2-4 BALB/c hosts 6 days after adoptive immunization against EL 4 tumor as described in "Materials and Methods." Counts represent total viable, nucleated cells.

‡  $^{51}\text{Cr}$ -labeled.

§ Figures represent immune percent lysis corrected for percent lysis of target cells incubated with normal BALB/c spleen cells (figure in parentheses).

|| Figures represent percentage of total cells taking up trypan blue in the immune cell-target cell mixture minus percentage of cells positive for trypan blue in the normal cell-target cell mixture (figure in parentheses). Normal cells were also purified by BSA-gradient separation before the assay.

### Apparent Absence of Nonspecific, Cell-Free Mediators of Toxicity in Direct, Cell-Mediated Cytotoxicity Reactions

Initial studies demonstrated that an immune lymphoid cell can destroy more than one target cell. Independent studies by Berke *et al.* (10) in a similar system also directly demonstrated that an immune cell can destroy more than one target cell. Target cell destruction, however, was not clearly limited to "direct contact mechanisms." As Dr. Perlmann emphasized earlier in this Conference (11) and elsewhere (12), a number of different pathways exist whereby target cells may be destroyed *in vitro* by immune lymphoid cells. High levels of cytotoxic activity (table 1) thus were examined to determine if they represented a maximum immune-cell effort *in vitro* utilizing all available pathways for cell-mediated cytotoxicity. Cytotoxicity provisionally was

interpreted to result from direct contact between presensitized lymphocytes and target cells. Additional studies, not presented here, argue against conventional, complement-dependent antibody contributing to cytotoxicity: Target cell destruction was not inhibited by carrageenan, an inhibitor of Cl; supernatants of immune-target cell reactions plus fresh guinea pig complement were not cytotoxic; nor did addition of fresh guinea pig complement facilitate target cell destruction by immune cells. To test for possible involvement of an additional pathway for cell-mediated cytotoxicity, the "innocent-bystander" experiment was utilized; it detects nonspecific cell-free toxins (13, 14). These amplifiers of target cell destruction are released from sensitized lymphocytes upon contact with specific antigen.

Immune spleen cells from adoptively immunized hosts were incubated simultaneously with  $^{51}\text{Cr}$ -

TABLE 2.—Specificity of target cell destruction by cytotoxic lymphoid cells\*

Experiment No.	BALB/c anti-EL 4 immune cells/ml	Target cells/ml		Labeled target cells killed† in 4 hours ( $\times 10^{-3}$ ) $\pm$ standard error of the mean
		$^{51}\text{Cr}$ -labeled	Unlabeled	
1	$5 \times 10^4$	$5 \times 10^5$ EL 4	$5 \times 10^5$ LSTRA	$40.0 \pm 1.1$
	$5 \times 10^4$	$5 \times 10^5$ LSTRA	$5 \times 10^5$ EL 4	$-1.1 \pm 1.5$
2	$10 \times 10^4$	$5 \times 10^5$ EL 4	$5 \times 10^5$ LSTRA	$47.5 \pm 5.0$
	$10 \times 10^4$	$5 \times 10^5$ LSTRA	$5 \times 10^5$ EL 4	$-8.5 \pm 1.0$

\*  $^{51}\text{Cr}$ -release cytotoxicity assay was described in "Materials and Methods" and in table 1 footnotes.

† Dead target cells were calculated by assuming "percent lysis" (see "Materials and Methods") approximates percentage of target cells originally present which have been killed.

labeled EL 4 cells and unlabeled LSTRA cells. EL 4 target cells were destroyed in the presence of innocent-bystander cells (table 2). If interaction of immune cells with EL 4 cells released nonspecific toxins whose actions spread within 4 hours beyond the immediate vicinity of the cell source, then we would expect to see damage to the LSTRA line. Reversal of  $^{51}\text{Cr}$  label, however, failed to show signs of LSTRA damage. Experiments not presented here show that LSTRA target cells readily release  $^{51}\text{Cr}$  when incubated with anti-LSTRA-immune cells. Within the confines of this immune-target cell system and the 4-hour incubation, high levels of cytotoxic lymphoid cell activity did not involve apparent pathways mediated by nonspecific toxins.

#### Direct, Cell-Mediated Cytotoxicity Without *In Vitro* Enlistment of Nonimmune Lymphoid Cells

Non immune lymphoid cells may contribute to cell-mediated cytotoxicity. One pathway would involve release of lymphocyte-dependent antibody from immune cells during the *in vitro* assay. For target cell destruction, lymphocyte-dependent antibody enlists nonsensitized lymphocytes which bind

antigen-antibody complexes (15), are theta negative (16), and presumably are thymus independent [see Perlmann (11)]. Other pathways would involve enlistment of nonsensitized lymphocytes or monocytes by antibody and complement secreted by immune cells during the *in vitro* reaction (17); secreted antibody could also enlist macrophages as cytotoxic effector cells (18). Utilization of these pathways during *in vitro* reactions would amplify cytotoxicity resulting from original immune killer cells acting alone.

Possible involvement of nonimmune cells in the present cytotoxicity system was examined by the dilution of immune cells in media alone or with nonsensitized syngeneic spleen cells. Nonimmune spleen cells from normal BALB/c donors were used directly without further purification. Immune spleen cells were enriched for cell-mediated cytotoxicity by an adoptive-transfer procedure. BSA-gradient cell separation was not done. Various concentrations of immune cells were incubated for 4 hours with  $^{51}\text{Cr}$ -labeled EL 4 target cells. The presence of additional nonimmune cells did not increase target cell destruction above levels found with immune cells alone (table 3).

TABLE 3.—Effect of nonsensitized lymphoid cells on cell-mediated cytotoxicity\*

BALB/c anti-EL 4 immune cells/ml	$^{51}\text{Cr}$ -labeled target cells/ml	Labeled target cells killed in 4 hours ( $\times 10^{-3}$ ) $\pm$ standard error of the mean	
		With normal† spleen cells	Without normal‡ spleen cells
$1.0 \times 10^4$	$5 \times 10^5$	$10.5 \pm 1.5$	$10.0 \pm 2.0$
$2.5 \times 10^4$	"	$17.0 \pm 1.0$	$23.0 \pm 1.0$
$5.0 \times 10^4$	"	$29.5 \pm 1.0$	$33.5 \pm 3.0$
$10.0 \times 10^4$	"	$51.0 \pm 1.0$	$73.5 \pm 10.5$

\*  $^{51}\text{Cr}$ -release cytotoxicity assay was described in "Materials and Methods" and in table 1 footnotes.

† Immune cells were serially diluted in normal BALB/c spleen cells at  $2 \times 10^6$  ml, thus maintaining the total (immune + normal), viable, nucleated spleen-cell count at  $2 \times 10^7$ /ml in all attacking cell mixtures.

‡ Immune spleen cells were diluted in media alone.



TABLE 4.—Viability of cytotoxic lymphoid cells during target cell destruction

Experiment No.	BALB/c anti-EL 4 immune cells/ml	EL 4 target cells/ml	Labeled cells dead in 4 hours ( $\times 10^{-3}$ ) $\pm$ standard error of the mean
1	$5 \times 10^4$ *	$5 \times 10^5$	$-1.1 \pm 0.8$
	$5 \times 10^4$	$5 \times 10^5$ *	$68.9 \pm 3.0^\dagger$
	$5 \times 10^4$ *	$5 \times 10^5$ *	$96.4 \pm 5.2^\dagger$
2	$5 \times 10^4$ *	$5 \times 10^5$	$-3.7 \pm 0.8$
	$5 \times 10^4$	$5 \times 10^5$ *	$27.5 \pm 2.0^\dagger$
	$5 \times 10^4$ *	$5 \times 10^5$ *	$24.0 \pm 2.0^\dagger$

\*  $^{51}\text{Cr}$ -labeled.

† Release of  $^{51}\text{Cr}$  from labeled immune cells incubated with unlabeled EL 4 was corrected for release of  $^{51}\text{Cr}$  from immune cells incubated in media alone. Calculation assumes "percent lysis" equals percentage of labeled cells originally present which have died.

‡ Release of  $^{51}\text{Cr}$  was corrected for release of  $^{51}\text{Cr}$  from labeled immune cells incubated with unlabeled EL 4 and for release of  $^{51}\text{Cr}$  from labeled EL 4 incubated with normal BALB/c spleen cells. Normal spleen cells were also purified by BSA-gradient separation.

The preceding evidence establishes that a cytotoxic lymphoid cell has the potential to destroy more than one target cell in a fashion apparently depending only on direct contact of the killer cell with the target cell. Other well-established pathways for cell-mediated cytotoxicity do not appear to be involved. This is encouraging because it suggests that, if other pathways for target cell destruction were brought into play, perhaps by the use of longer incubation periods or different sources of immune cells, then additional target cells would be destroyed.

### Viability of Immune Cells During Target Cell Destruction

Viability of attacking cells during target cell destruction was examined with the use of  $^{51}\text{Cr}$  release as an index of viability, under the assumption that attacking cells label with  $^{51}\text{Cr}$  and release  $^{51}\text{Cr}$  if damaged. Immune spleen cells, enriched for cell-mediated cytotoxicity by an adoptive-transfer procedure and subsequent BSA-gradient purification, were labeled with  $^{51}\text{Cr}$  and incubated with unlabeled EL 4 target cells. Attacking cell damage was not significant (table 4). Reversal of label to the target cell again demonstrated that the average immune cell can destroy more than one target cell during a 4-hour incubation (table 4, expt. 1). Labeling of attacking cells did not conflict with the cytotoxic activity of these cells. Why immune activity was increased in experiment 1 after attacking cells were labeled with  $^{51}\text{Cr}$  is not clear.

### DISCUSSION

Experiments presented here deal with a very limited aspect of cytotoxic lymphoid cell regulation. They illustrate high levels of cytotoxic cell ac-

tivity obtained by immunizing presensitized cells in an X-irradiated host, enriching activity with BSA-gradient cell separation, and assaying activity *in vitro*. These procedures yield high levels of cytotoxic activity presumably by avoiding the normal host environment and its suppressive regulators of cell-mediated cytotoxicity.

Finding that a cytotoxic cell, without destroying itself, may destroy more than one tumor cell *in vitro* complements data of Brunner *et al.* (19) who demonstrated that cytotoxic immune cell function is not diminished by tumor cell interaction. A cytotoxic immune cell, from these results, would appear to have the functional capacity to cause widespread damage within a tumor mass; at least more tumor destruction would result than in a situation wherein the average immune cell died after tumor cell destruction. Indeed, preliminary results demonstrated a significant *in vivo* function of cytotoxic lymphoid cells used for passive immunization (20). Disagreement, however, persists regarding the fate of cytotoxic cells. The concept of attacking cell death after target cell interaction is either refuted (19, 21–23) or supported (24, 25) by *in vitro* assessment of cytotoxicity reactions with microscopic observation or kinetic analysis of target cell destruction. These conflicting positions may have come about in part from existence of multiple pathways for cell-mediated target cell destruction.<sup>4</sup>

<sup>4</sup> At least 4 types of cytotoxicity mediated by lymphoid cells have been identified (12). 1) Sensitized lymphocytes destroy target cells directly. 2) Nonsensitized lymphocytes destroy target cells coated with lymphocyte-dependent antibody. 3) Nonsensitized lymphocytes and monocytes destroy target cells coated with antibody and the first 7 components of complement. 4) Sensitized lymphocytes stimulated by antigen, or nonsensitized lymphocytes mitogenically stimulated, release mediators which nonspecifically destroy target cells. Immune lymphoid cells, in addition, may arm macrophages for target cell destruction (18).

Different "attacking" cell types are associated with individual cytotoxic pathways, and one or more of the attacking cell types may die after tumor cell interaction.<sup>5</sup>

The issue is further complicated by *in vitro* incubation times associated with assessment of cell-mediated cytotoxicity. Long incubation periods (days) may permit several pathways of cytotoxicity and may be adequate for *in vitro* maturation of inactive precursor cells to killer cells (25). Short incubation periods (hours), used in the present study, restricted cytotoxicity to pre-existing sensitized cells which appeared to damage target cells only by direct contact. A final set of variables affecting the apparent fate of cytotoxic immune cells includes clumping of attacking cells, pH changes, nutrient depletion, and target cell coating by noncytotoxic antibody. These factors may nonspecifically destroy cytotoxic cells or functionally inactivate cytotoxic cells in a reversible fashion.

An appealing goal is manipulation of the environment of the tumor-bearing host to permit the development and function of highly active cytotoxic lymphoid cells such as artificially created here. Encouraging are the findings that cytotoxic lymphoid cells, as identified in the present study, can function in a tumor-bearing host if supplied exogenously (20) and that manipulation of tumor immunogenicity will increase *in vivo* development of cytotoxic lymphoid cells (8). Certain pertinent reminders, however, are: 1) Tumors may differ in susceptibility to various immune pathways for destruction; 2) unsuppressible regulators *in vivo* may forbid natural operation of cytotoxic pathways which have been identified *in vitro*; and 3) the present experimental system involves alloantigens, not tumor-specific antigens, and involves a target tumor cell highly susceptible to lysis.

*Note added in proof:* Increased immunogenicity of EL 4 in C57BL/6 mice by Con A-coating tumor cells may depend on the line of EL 4. Preliminary studies with EL 4 kindly supplied by Dr. E. A. Boyse have failed to demonstrate an effect of Con A on immunogenicity.

<sup>5</sup> Nomenclature for "cytotoxic cells" is in a state of flux, since distinct subpopulations are being identified. "Cytotoxic lymphoid cells" measured in the present study presumably are synonymous with "cytotoxic lymphocytes" identified by Brunner *et al.* (19). We have not, however, attempted to identify classes of attacking cells associated with direct cytotoxicity observed in the present study.

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# Allogeneic Cell-Mediated Testing for Human Tumor Antigens<sup>1, 2</sup>

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**SUMMARY**—With the use of the microassay for cell-mediated immunity, specific reactivity was demonstrated against a target cell derived from an epidermoid carcinoma of the larynx with lymphocytes from patients with larynx and other head and neck cancers. Detection of these antigens was partially obscured by excess reactivity from specific and nonspecific causes unrelated to cancer. To better understand these effects, we examined reactions against histocompatibility and other non-tumor-associated antigens and the nonspecific effect from granulocyte contamination of lymphocyte-effector cell samples.—*Natl Cancer Inst Monogr* 35: 251–257, 1972.

THE RECENT demonstrations by several investigators (7–9) of a cell-mediated, tumor-specific reactivity in human cancer and the sharing of antigens between tumors of the same histologic type (10) require confirmation on a large scale. If human tumor antigens exist and are shared according to some classification, groups of highly reacting patients should be identified and specific reactivity should be found within groups of cancers.

When allogeneic human systems are tested, however, reactions are not confined to only tumor-associated antigens but also to interferences from other antigenic systems. For successful identification of tumor-associated antigen (5) reactions must be sorted to exclude background, specific reactivities against other antigens, and nonspecific effects. This study reports on an antigen associated with epidermoid cancers of the larynx, and perhaps for all

head and neck epidermoid cancers, on antigens for breast (10) and colon cancers (5, 6), and on the problems encountered.

## MATERIALS AND METHODS

*Cultured target cells.*—Short-term culture and established cell lines were used for both cell-mediated and serologic testing. Besides cultures grown from tumors in our laboratory, target lines were received from Dr. E. Bloom (University of California at Los Angeles), Dr. E. Plata (National Cancer Institute Bethesda, Md.), and Dr. W. Nelson-Rees (Naval Biological laboratory, Oakland, Calif.). All cultured target cells were tested for mycoplasma contamination by Dr. Leonard Hayflick (Stanford Medical School, Palo Alto, Calif.) and in our laboratory.

*Microassay for cell-mediated immunity (MCI).*—The assay was described previously (11, 12). For adaptation to human systems, the number of target cells was reduced to 100–300. The test tray (Microtest plate, Falcon 3034, Oxnard, Calif.) was flooded with 5–7 ml medium after sufficient time had passed so that added lymphocytes had settled, to lessen excessive reduction of target cells. After incubation for 2 days the test was terminated; the wells were washed with Hanks' medium, fixed, and stained. Surviving target cells were electronically

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TABLE 1.—Results of tests on cultured target cell 287 from an epidermoid cancer of larynx: Scores with lymphocytes from patients with various cancers

MCI score range	Larynx	Head and neck (other than larynx)	Normal
0-50	0 1 45	0 0 26 50	0 0 0 0 0 19 40 45
51-100	63 66 67 81 89	54 70 86 91 100	54 98
101-300	102 158 177 200 214	117 118 124	115 145 158 184 278 282
301-600	302 411 432 464 473 521	330 392 405 523 547	301
	Gynecological	Breast	Lung
0-50	1 22 24 35 40	0 0 2 16 30 33 35 37 43	0 0 0 0 1 5
51-100	53 73 80 81 88 94	93 123	55 77
101-300	114 130 136 265	112 191 195	104 155 194 208 213
301-600		310 323 428 600	301 512
	Colon	Prostate	Bladder
0-50	0 1 15 24 46 43	0 0 16 19 26	0 0 33 50
51-100	65 71 93 95	76 86	
101-300		161	246 280
301-600	312 383	403 404 446 600	588
	Others		
0-50	0 0 0 0 0 1 1 9 20 21 42 47 50		
51-100	53 56 60 98		
101-300	103 115 122 169 222 273		
301-600	335 355 383 431		
601-1000	605		

counted by image analysis (Imanco, England). Each sample was tested in triplicate with 4 having titrations starting at 50,000 cells. Results were compared with a medium control. The percent target cells destroyed in the 4 titrations, beginning with the most concentrated cells, were designated *a*, *b*, *c*, and *d*; a final score was derived by the following formula:

MCI score = 1*a* + 2*b* + 3*c* + 4*d*.

Greater weight was thus given to target cell reduction by lower numbers of lymphocytes to separate strong reactions from nonspecific background and to rank the strength of reactions.

*Cytotoxic plating inhibition (CPI) test.*—The test will be described in greater detail elsewhere. Briefly, 2 μl of antiserum was placed in the wells of a microtest plate under 5 μl of sterile mineral oil; 300–400 cultured cells were added, and then 5 μl rabbit complement (diluted 1:4 in medium) was added one-half hour later. The test was placed in a moisturized CO<sub>2</sub> incubator overnight and was

stopped the following morning by the addition of 10 μl neutralized 40% formalin. A glass slide was placed over the wells, and the plate sealed with hot vaseline. The test was scored on a scale from 1–8 by readers under an inverted phase-contrast microscope.

RESULTS

Lymphocytes from 161 patients with various cancers were tested on target cell 287 from a woman with epidermoid carcinoma of the larynx. Table 1 shows the raw scores with groups divided by type of cancer. Positive reactions were considered to be scores >50; 16 of 19 samples from patients with cancer of the larynx reacted against the target cell, whereas only 9 of 17 samples from normal individuals reacted against the same target cell. Patients with other head and neck cancers were the only other group that approached this range of reactivity.

The complete results of testing on target cell 287



TABLE 2.—Results of tests on cells cultured from epidermoid cancers of larynx

Target cell	Type of cancer	Number of samples	Positive reactions (>50)	Mean score	Standard error	<i>t</i> score compared to:		<i>P</i>	
						Normal	Other		
287	Larynx	19	16	203.5	40.2	2.14	1.49	<0.05	<0.2
	Head and neck (other than larynx)	17	13	178.4	44.5	1.50			
	Head and neck (all)	36	29	191.6	29.5	2.30		<0.05	
	Gynecological	15	10	82.4	16.7	1.38			
	Breast	18	9	135.2	35.5	0.77			
	Lung	15	9	121.7	37.8	0.36			
	Colon	12	6	95.7	35.5	0.12			
	Prostate	13	7	172.7	59.1	1.10			
	Bladder	7	3	166.7	78.6	0.79			
	Others	28	15	127.5	30.4	0.66			
	Normal	17	9	101.1	25.9				
296	Head and neck (all)	28	20	181.1	34.6	2.18		<0.05	
	Normal	11	5	81.9	29.7				
315	Head and neck (all)	22	14	99.9	23.8	0.81	2.31		<0.05
	Normal	4	1	54.6	50.8				
	Others	11	4	37.5	12.7				

are summarized in table 2. A comparison of mean scores between patients with larynx cancer and normal individuals was statistically significant by the *t* test at the 0.05 level. The mean score for all head and neck cancers, including carcinomas of the larynx, was also significant; but if carcinomas of the larynx were excluded from this group, the difference was no longer significant.

The indication from target cell 287 that an antigen associated with head and neck cancer existed

received support from less complete results obtained from 2 other cell cultures derived from epidermoid carcinomas of the larynx. Significant differences were observed when results with all head and neck cancers were compared with those with normal controls on target cells 296 and 315. In the latter, tests with lymphocytes from normal individuals were too few, and results were compared with other cancers.

To demonstrate specific reactivity in other hu-

TABLE 3.—Results of tests on cultured cells from other types of cancers

Target cell	Type of cancer	Number of samples	Positive reactions (>50)	Mean score	Standard error	<i>t</i> score compared to:			<i>P</i>
						Normal	Other		
464 (breast)	Breast	21	13	118.4	30.4	3.07	2.04	<0.01	<0.05
	Lung	5	2	63.2	40.3	1.02			
	Prostate	5	2	33.0	14.1	0.71			
	Bladder	4	0	27.3	9.3	0.48			
	Others (all)	35	14	51.5	12.0				
	Normal	9	2	21.0	9.0				
373 (colon)	Colon	7	3	199.7	109.4	1.29			
	Gastric	6	5	171.3	96.1	1.17			
	Gastrointestinal (includes above)	13	8	186.6	70.8	3.65	0.89	<0.01	
	Breast	9	2	51.3	22.9	0.49			
	Head and neck	13	6	119.5	41.9	1.25			
	Others	12	4	107.4	53.2				
	Normal	7	2	52.0	33.5				
	Lung	12	7	158.5	62.8		0.76		
270 (lung)	Others (all)	24	12	105.8	29.2				

TABLE 4.—Exclusion of nonspecific data in tests with target cell 287

Type of cancer	Number of samples	
	Tested	Excluded
Larynx	23	4
Head and neck (other epidermoid cancers)	23	4
Head and neck (all epidermoid cancers)	44	8
Gynecological	19	4
Breast	23	5
Lung	19	4
Colon	18	6
Prostate	17	4
Bladder	8	1
Others	31	3
Normal	21	4
Total	200	39

man tumor systems, target cells from other cancers were tested. Table 3 summarizes the results for 3 other target cells derived from carcinomas of the breast (464 = Ht39f), colon (373 = Ht29), and lung (270 = C54). In each case, the highest mean score was observed for the same type of cancer as the target cell. With 270, the mean score appeared higher than that of other cancers, but the difference was not significant. Here too, data were not sufficient with normal cells.

The profile of reactors within each group (table 1) showed the excess positive reactions which interfered with the detection of clear specificities. Also,

among those tested were some patients and normal individuals who reacted against most of the target cells used. Obviously, if most target cells regardless of their source are destroyed, the specificity of the reaction cannot be determined and the result must be excluded. The criterion used for exclusion of results from one lymphocyte sample was that the cell reacted strongly against all or at least 3 of 5 target cells tested. Most samples reacted against all target cells. Positive results, in which the lymphocyte sample was tested on only 1 or 2 targets, were not included, since information was not sufficient to conclude that the reactions were specific. The number excluded per group is shown in table 4; it averaged roughly 1 in 5 tests. Further studies were then carried out to explain the reasons for the exclusion of data.

Since reactions against histocompatibility antigens are most likely in allogeneic testing, the effect of transfusions to patients was studied. The transfusion record for patients was examined, and 16 patients were found who had been transfused with one or more units; most of these patients had received blood within the last 3 months before the test was performed. The MCI scores for these patients were compared with those for nontransfused patients tested on the same tray and thus against the same target cells. The results (table 5) showed a significant difference between the 2 groups.

Antigens other than histocompatibility and tu-

TABLE 5.—Comparison of cell-mediated reactions among patients after transfusions

Number of target cells used	Patient No.	Transfused patients			Nontransfused patient tested on same tray	
		Units received	Mean score	Standard error	Mean score	Standard error
8	44384	4	60.6	40.0	37.8	20.7
6	44681	3	31.8	13.3	21.8	8.4
5	44530	2	114.0	65.3	50.6	23.3
8	44628	6	150.1	35.9	118.8	26.4
8	44634	1	83.7	29.7	118.8	26.4
5	44157	2	179.2	70.8	96.4	37.8
6	44161	1	151.3	47.7	88.6	19.8
7	44251	1	152.1	43.4	52.3	26.0
9	44282	8	58.7	21.1	32.1	20.3
34	44386	4	103.6	21.8	83.4	21.6
15	44444	1	119.3	47.1	35.8	21.6
4	44523	4	193.8	71.1	95.5	21.4
10	44277	3	9.7	9.3	46.3	17.7
11	44278	2	130.1	52.0	157.1	70.1
4	44155	2	89.8	32.7	45.8	22.3
12	44254	2	186.9	69.1	146.3	59.8
Average.....			113.4	13.8	76.7	10.6
		<i>t</i> = 2.11	<i>P</i> < 0.05			

TABLE 6.—Detection of an antigen on cultured cells by the CPI method\*

Target cell	Derived from	Serum No.						
		B2135	B2157	37332, breast cancer	source 39660, cancer of tongue	39867, colon cancer	39813, colon cancer	39861, breast cancer
		Normal						
122 (HeLa)	Cervix cancer	++	++	++	++	+++	+++	+++
123	Embryo lung	++	++	+++	+	++	++	+++
124	Embryo lung	+	—	+	+	++	++	++
462 (G5R)	Breast cancer	—	+	—	—	++	+++	++
463 (G511)	Breast cancer	++	++	++	++	+++	++	+++
224	Melanoma	—	—	—	—	—	—	—
250	Sarcoma	—	—	—	—	—	—	—
287	Larynx cancer	—	—	—	—	—	—	—
373	Colon cancer	—	—	—	—	—	+	—
419	Epidermoid cancer	—	—	—	—	—	—	—
495	Ovary cancer	—	—	—	—	—	—	—
496 (Al Ab)	Breast cancer	—	—	—	—	—	—	—
374	Prostate cancer	—	—	—	—	—	—	—
470	Melanoma	—	—	—	—	—	—	—
125 (HEp-2)	Larynx cancer	—	—	—	—	—	—	—
157	Mesothelioma	—	—	—	—	—	—	—
158	Melanoma	—	±	—	—	—	—	—
296	Larynx cancer	—	—	—	—	—	—	—
329	Larynx cancer	—	—	—	—	—	—	+

\* + + +, 2/2 very strong positive reaction; + +, 2/2 positive reaction; +, 1/2 positive reaction; ±, 1/2 weak reaction; and —, 0/2 positive reaction.

mor antigens may participate in cell-mediated reactions. Through serologic screening with the CPI test on a battery of cultured cells used as targets in cell-mediated tests, an antigen or antigens shared by 5 established lines were determined with 7 sera from patients and normal individuals (table 6). The sera identifying these antigens were analyzed for human histocompatibility antigens (HL-A) by tests on lymphocytes with known HL-A groups. The negative results on lymphocyte testing and the identification of the antigen on HeLa cells, which especially resist HL-A typing, suggested that the antigens were not HL-A in origin. On the other hand, the source of lymphocytes and the derivation of the cultured lines indicated that the antigens were not related to cancers. Nevertheless, the antigens were found on target cells and hence must be studied in their relationship to cell-mediated effects. The results from 20 patients tested on HeLa cells and at least one other cell line sharing the antigen are shown in table 7. Based on a positive-negative criteria of MCI scores above and below 50, only 8 cases of discordance were observed. In each case, HeLa cells were more sensitive to testing than other cells sharing these antigens, but, in general, cell-mediated testing paralleled the serologic results.

Technical difficulties in the preparation of puri-

fied lymphocytes and the presence of contaminating granulocytes were important factors in cell-mediated tests. Lymphocytes and granulocytes were separated by Ficoll-Isopaque flotation (12). Both

TABLE 7.—Comparison of MCI scores on target cells sharing an antigen detected by CPI test

Patient No.	MCI scores on:				
	122 (HeLa)	123	124	462 (G5R)	463 (G511)
41576	650	449	790	413	
41577	717	673	944	484	
41579	790	636	823	577	
41580	927	674	951	643	
42081	81			58	
42086	77			0	
42088	170			79	
42092	275			60	
42306	456			3	263
42308	324			0	121
42311	540			12	395
42313	262			0	240
42194	0				0
42295	295				542
42354	817				255
42358	345				26
42368	345				83
42370	526				171
42390	299				35
42402	185				41
Concordance with 122		4	4	8	9
Discordance with 122		0	0	5	3



TABLE 8.—Effect of granulocyte contamination on target cells

Target cell	MCI score at (lymphocyte/granulocyte ratio)				
	100/0	75/25	50/50	25/75	0/100
154	13	50	56	62	113
125 (HEp-2)	53	158	239	432	432
115	10	10	0	20	168
158	122	159	329	413	364
122 (HeLa)	38	8	56	134	94
147	34	351	182	248	511
125	0	465	796	980	1000
123	37	76	95	311	546
203	205	388	265	183	218
166	15	84	118	155	203
159	4	332	192	184	175
Mean	51.6	189.2	211.6	283.8	347.6

lymphocyte preparations obtained from the interface and granulocytes from the pellet were contaminated with <10% of the other cell. These cells were tested in the purified state and in mixtures on 9 different target cells (table 8). Granulocytes and target cell reduction were correlated. Long-established lines were usually more sensitive to the granulocyte effect. For daily tests of lymphocytes against cultured tumor cells, preparations with >10% granulocytes were not used. Fortunately, most lymphocyte preparations at this level gave CMI scores <50 within the background allowed.

DISCUSSION

Lymphocytes from patients with epidermoid cancer of the larynx reacted specifically against cells cultured from the same type of tumor. A higher frequency of strong reactors was also found among other patients with squamous cell carcinomas of the head and neck, but results were not significant. When both groups were combined, however, results were significant, which suggests that all epidermoid cancers of the head and neck share antigens. Supporting evidence was derived from tests on 2 other cultured cells from the same type of cancer; these tests gave similar results. To ascertain whether a single or several antigens are completely or partially shared by head and neck cancers requires testing of sufficient numbers of patients from the group on target cells from various head and neck cancers.

Differences were also significant in adenocarcinomas of the breast and of the colon, which agrees with the observations of the Hellströms (5, 6, 10).

What appeared to be a difference in tests on a lung cancer target cell was not significant. In tests with several other target cells, specificity, although suggested, was not significantly demonstrated. With still other target cells, no specificity was found, which suggested that cells other than tumor cells had been cultured.

These data and the exclusion of results illustrate the difficulties in allogeneic testing for human tumor antigens. The major difficulty is due to hyper-reactivity of lymphocyte samples and excessive cell reduction by patients with other types of cancer and by normal individuals. To find specificity in such systems, reactions against antigens other than tumor-specific antigens must be recognized and ruled out.

Reactions against histocompatibility antigens on tumor cells in culture are partly responsible for the excessive reactivity, since patients may be sensitized from transfusions and from pregnancy. Of 48 patients whose records were examined for transfusions, 16 patients had received recent transfusions. When MCI scores were compared with those from patients who were not transfused and who were tested on the same tray, differences were significant, indicating that these reactions must be considered. Sera from cancer patients were also analyzed for HL-A activity; 5–10% reacted against a panel of lymphocyte samples. The simplest method of recognizing and eliminating transplantation reactions is to test lymphocytes from a patient on a series of target cells preferably typed for HL-A antigens and derived from the same type of tumor. Because of the polymorphism of the human histocompatibility system, it would be unlikely that the same HL-A or other histocompatibility antigens would be represented on all the target cells.

Reactions against antigens other than tumor or histocompatibility antigens were found with the CPI test. Extensive screening of sera from patients revealed an antigen on cultured lines. Later sera from normal individuals also reacted with the same target cells. Since 2 of the target lines were not from cancers, these reactions were not directed toward tumor antigens. Moreover, it was unlikely that the antisera involved HL-A specificities, since they were negative against 80 lymphocyte samples. Because of the presence of these antigens on membranes of cultured target cells, MCI tests on cell lines sharing this antigen were compared, and the results from these tests approximated the serologic

results. A more thorough study is needed of all antigens on cultured target cells which may possibly participate in cell-mediated reactions.

Finally, technical problems still plague *in vitro* tests on cell-mediated immunity. Pure lymphocytes are often difficult to isolate in sufficient numbers. Contamination with granulocytes causes significant target cell reduction and must be controlled, especially for more sensitive target cells (13). Possibly, both lymphocytes and granulocytes are required to mediate specific target cell destruction, and our results are weakened by the absence of one component of the effector mechanism. However, the specificity of cell-mediated reactions appeared more closely related to the source of lymphocytes than to the source of granulocytes. Although a mixture with a given proportion of lymphocytes and granulocytes may be more efficient in cell-mediated immunity, in practice this was more difficult to control.

Thus human cancers possessed antigens associated with similar types of cancers, but the detection of these antigens by allogeneic testing was clouded by interference from reactivity against histocompatibility and other antigens. Characterization of the cell-surface antigens on target cells should help in understanding these reactions, so that a clearer detection of tumor-associated antigens may become possible.

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# Cellular Immune Reactions to Human Leukemia<sup>1, 2</sup>

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**SUMMARY**—The cellular immune response to antigens associated with human acute leukemia was studied by 3 different assays: 1) skin tests for delayed hypersensitivity to tumor cell extracts, 2) mixed lymphocyte culture (MLC), and 3) lymphocyte cytotoxicity assay (LCA). Although some results in each of these assays were positive, they did not correlate well with each other. Skin tests with autologous membrane extracts of blast cells were correlated with disease state. They were positive in 84% of patients tested during remission and in only 23% of patients tested in relapse. In serial tests on several patients, skin reactivity changed from positive to negative at the time of clinical relapse. The results in MLC did not correlate with disease state, but did correlate well with the time after cessation of chemotherapy, with most patients having a peak in reactivity to autologous blast cells at 10–20 days. Reactivity of patients in LCA also did not correlate significantly with disease state, though there was a tendency toward more reactivity during relapse. Some family members of patients with leukemia and unrelated adults also had reactivity against the cells of leukemia patients in LCA. These results indicate that the various assays for cellular immunity may be measuring reactivity to different leukemia-associated antigens or measuring different phases of the immune response.—*Natl Cancer Inst Monogr* 35: 259–266, 1972.

THERE IS increasing evidence that human acute leukemia cells have tumor-associated antigens.

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Cellular immune reactions to these antigens have been detected in a variety of assays. Delayed-hypersensitivity reactions have been elicited by membrane extracts of autochthonous leukemia cells (1, 2). In mixed lymphocyte cultures (MLC) leukemia cells have produced some stimulation of autochthonous (2–5) and HL-A-compatible lymphocytes (6). Autochthonous and allogeneic lymphocytes have also been shown to be cytotoxic to the cells of patients with acute leukemia (2, 7, 8).

It is generally believed that cell-mediated immunity is of central importance in host resistance against tumors and in suppression of tumor growth (9–11). It is also often assumed that a particular assay of cellular immunity is a measure of *in vivo* resistance against tumor growth. *In vitro* cytotoxicity

city reactions have been particularly stressed as likely analogues of the cell-mediated defense against tumors (11-13).

Some recent observations in our laboratories have necessitated a re-evaluation of these assumptions. When cellular immunity to human leukemia-associated antigens was studied by 3 different assays, we found that the results of the individual assays correlated poorly with each other. Only the skin tests for delayed hypersensitivity correlated with the clinical state of the patients.

This paper extends the earlier studies and characterizes the reactivity found in each of 3 immunologic assays. In addition, it further examines the relationship of the assays to clinical events.

## MATERIALS AND METHODS

*Subjects studied.*—All patients had acute leukemia and were treated at the National Cancer Institute. There were 52 with acute lymphocytic leukemia (ALL) and 28 with acute myelogenous leukemia (AML). Relatives of the patients and unrelated normal adults were also studied in the *in vitro* assays.

*Target cells for in vitro assays.*—As previously detailed in (2, 8), leukemia blast cells were collected from the peripheral blood of patients by venipuncture or by leukapheresis, or from the bone marrow. Peripheral blood was collected in 5-20 U/ml of heparin (Upjohn) and bone marrow in 20 U/ml of heparin. After sedimentation of the erythrocytes by dextran or on Ficoll isopaque gradients (14), the white blood cells were washed and suspended at a concentration of  $1 \times 10^7$ /ml in culture medium. Some assays were performed with fresh target cells, within 18 hours of collection. However, most assays were performed with target cells that had been stored frozen in liquid nitrogen (2).

*Lymphocyte cytotoxicity assay (LCA).*—Peripheral blood lymphocytes from patients and normal controls were isolated on Ficoll Hypaque gradients (14). Effector or attacker cells were prepared by the resuspension of nonlabeled lymphocytes in Eagle's minimal essential medium with 10% heat-inactivated fetal bovine serum and 200  $\mu$ g/ml carra-genan, a Cl complement inhibitor (15). Target cells were labeled with  $^{51}\text{Cr}$  after erythrocytes had been removed by ammonium chloride Tris (ACT) lysis (8). Quadruplicate mixtures of  $3 \times 10^6$  effector lymphocytes and  $5 \times 10^4$  labeled target cells (60 : 1 effector to target cell ratio) were made. The

cells were incubated for 4 hours at 37°C on a rocker platform (8, 16). The amount of cell lysis was determined by the counts per minute (cpm) of  $^{51}\text{Cr}$  released into the supernatants. The percent lysis was calculated relative to the maximum releasable  $^{51}\text{Cr}$ , determined by samples of labeled target cells being frozen and thawed 4 times:

$$\% \text{ lysis} = \frac{\text{cpm } ^{51}\text{Cr released in experimental group}}{\text{cpm } ^{51}\text{Cr released from cells by } 4 \times \text{freeze-thaw}} \times 100.$$

Controls for background release of  $^{51}\text{Cr}$  for each experiment included incubation of labeled target cells with autologous target cells that were not labeled or treated with ACT. Background release in these experiments averaged 15%, with a range of 6-45%. Experimental results were expressed as corrected percent lysis, by the subtraction of the background  $^{51}\text{Cr}$  release. Differences between experimental and control groups were analyzed for significance with Student's *t* test. A test was considered positive if the *P* value was  $<0.05$ .

*MLC.*—MLC was performed as previously described (2). The stimulating cells were treated with mitomycin C, and the degree of stimulation of the responding cells was estimated by the amount of  $^3\text{H}$ -thymidine incorporated after incubation for 5-7 days. The ratio of responding cells to stimulating cells was 2 : 5. The results were expressed as the ratio of stimulation: disintegrations per minute (dpm) in cultures stimulated with the cells under study/dpm in cultures of cells stimulated by autologous normal peripheral lymphocytes, *i.e.*,

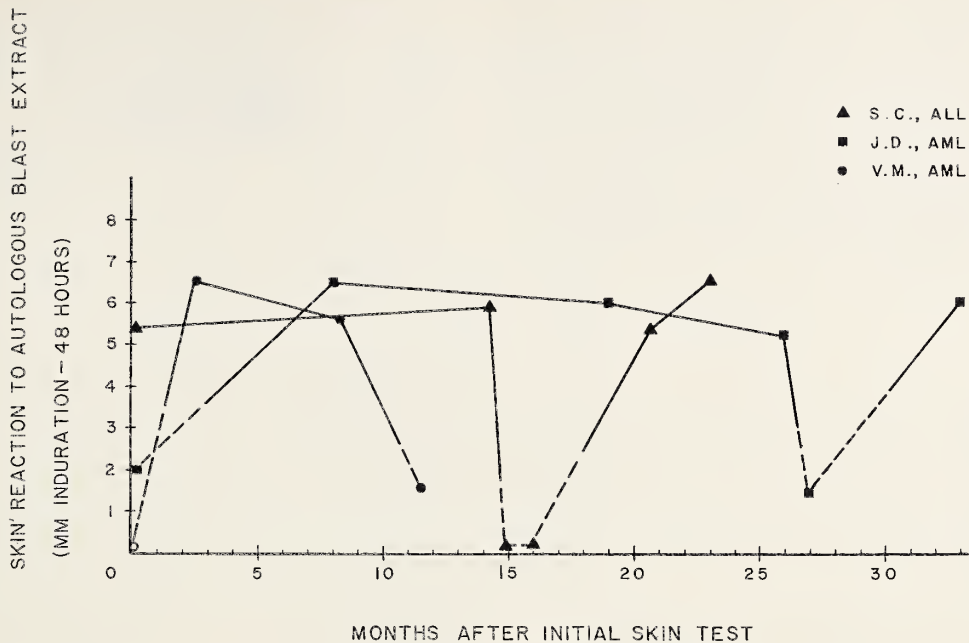
$$\frac{A + X_m}{A + A_m}.$$

Reactions were defined as positive when

the ratio was  $\geq 2$ .

*Skin tests.*—Leukemia blast cells and remission cells were harvested from heparinized peripheral blood or bone marrow and suspended in isotonic saline (2). Membrane extracts were then prepared as previously described (1).

Skin tests were performed by intradermal inoculation of 0.1 ml of the membrane preparation, usually on the patient's back. The tests were done 5-30 days after cessation of treatment with chemotherapeutic agents, when the patients were in clinical remission, or at the time of bone marrow relapse. All tests reported here were performed at a protein concentration of 1 mg/ml. To assess the ability of the recipient to manifest a delayed skin



TEXT-FIGURE 1.—Skin tests with membrane extracts of autochthonous leukemia cells, performed at various times after initial skin tests. *Solid lines* indicate periods of clinical remission. *Dashed lines* indicate periods of relapse.

reaction, tests were performed with a battery of 5 recall antigens (2). Four patients, who did not react to any of these microbial antigens, were considered anergic and were excluded from the study.

Skin tests were examined at 24 and 48 hours after inoculation of the preparation, and measurements made with a Vernier caliper. A positive reaction was defined as induration of  $\geq 5$  mm at 48 hours.

## RESULTS

### Skin Tests

Most skin tests were performed with membrane extracts of autochthonous cells. When patients with ALL were tested with leukemia cell preparations during clinical remission, a high incidence of reactivity was observed (table 1). In contrast, patients tested while in bone marrow relapse were usually

unreactive; only 1 of 16 tests was positive. The observed positive reactions appeared to be quite specific, with remission membrane preparations producing uniformly negative results. In 9 of these tests with remission extracts, simultaneous tests with leukemia cell extracts were positive.

The tests in patients with AML gave similar results (table 2). Most patients in remission had positive reactions to the blast extracts (18/20). Patients in relapse had a significantly lower proportion of positive reactions (7/19). As in the ALL patients, membranes of autochthonous remission cells did not elicit positive reactions.

Eighteen patients were tested at least twice. There was no indication that the initial skin tests produced sensitization against the leukemia-associated antigens. The incidence of reactivity in the first tests and in subsequent tests was similar. In 15

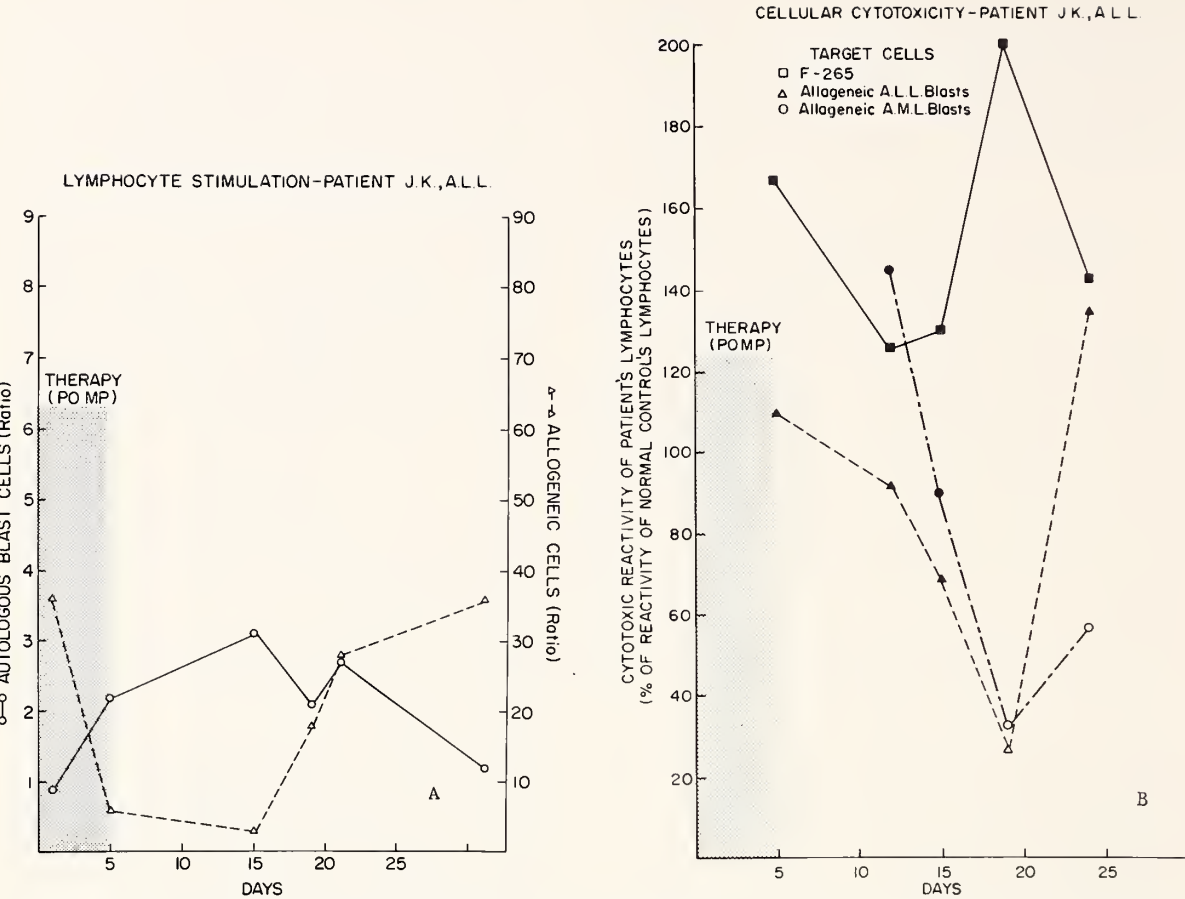
TABLE 1.—Skin tests of patients with ALL with membrane extracts of autochthonous cells

Test material	Tests positive/total No. of tests	
	Remission	Relapse
Leukemia cell membranes	18/23	1/16
Remission cell membranes	0/14	0/5

TABLE 2.—Skin tests of patients with AML with membrane extracts of autochthonous cells

Test material	Tests positive/total No. of tests	
	Remission	Relapse
Leukemia cell membranes	18/20	7/19
Remission cell membranes	0/5	0/4





TEXT-FIGURE 2.—Effect of chemotherapy (prednisone, vincristine, methotrexate, and 6-mercaptopurine) on reactivity of ALL patient in MLC and LCA. A: tests in MLC with autochthonous blast cells and with the same allogeneic cells. B: tests in LCA against allogeneic blast cells and against the human lymphoid cell line, F-265. Results are expressed as the percent of reactivity of patient compared to that of the normal control. Closed symbols indicate significantly positive results ( $P < 0.05$ ), and open symbols indicate results which were not significant.

TABLE 3.—Skin tests with membrane extracts of allogeneic cells

Disease of recipient	Type of extract	Number of patients with positive tests/total No. of patients tested
ALL, remission	ALL blasts	19/31
	ALL remission leukocytes	0/10
	AML blasts	0/8
	Normal leukocytes	0/8
AML, remission	AML blasts	3/8
	AML remission leukocytes	0/5
	ALL blasts	0/6
	Normal leukocytes	0/5
Normal	Normal leukocytes	0/23

patients, all the serial test results correlated well with the clinical state. Tests were positive for patients in remission and were negative for patients in relapse. Text-figure 1 shows representative results for 3 patients, in whom 4–6 tests were done.

We recently conducted tests with membrane extracts of cells from allogeneic leukemia patients. Each recipient was inoculated with 3 or 4 preparations. Whenever possible, tests were done with extracts of the blast cells and remission cells from the same patient. The results are summarized in table 3. Reactions were positive in both ALL and AML patients, in response to extracts of blast cells from the same disease. However, the incidence of reactivity was lower than that seen with autochthonous extracts. In addition, most of the reactive patients

TABLE 4.—Lymphocyte cytotoxicity reactions against autochthonous target cells

Attacking lymphocytes	Tests positive/total No. of tests (%)	
	Target cells	
	Blasts	Normal
ALL	8/20 (40)	0/22 (0)
AML	6/19 (32)	0/10 (0)
Normal		0/30 (0)

were positive to only 1 of the test preparations. The antigens recognized in these tests appeared to be leukemia associated, since extracts from remission leukocytes and from leukocytes of normal individuals gave negative reactions. Allogeneic extracts did not elicit any positive reactions in 23 normal controls. This lack of reactivity to allogeneic normal cell extracts was somewhat surprising, since, in many combinations, there were known HL-A antigenic differences.

### LCA

Reactivity of patients with acute leukemia and of normal individuals against autochthonous target cells is summarized in table 4. Results were positive in 8 of the 20 tests with ALL blasts and with 6 of 19 tests with AML blasts. Reactions were negative with remission target cells. All tests of normal controls against their own leukocytes were negative.

The reactions against the blast target cells did not correlate with clinical state. There was at least as much reactivity during bone marrow relapse as there was when the patients were in remission.

TABLE 5.—Lymphocyte cytotoxicity reactions against allogeneic target cells

Target cells	Tests positive/total No. of tests (%)		
	Attacking lymphocytes		
	Normal	ALL patients	AML patients
Normal lymphocytes	5/220 (2.3)	0/20 (0)	0/15 (0)
ALL blasts	55/117 (47)	9/23 (39)	4/15 (27)
ALL remission lymphocytes	15/79 (19)	0/5 (0)	0/2 (0)
AML blasts	40/95 (42)	6/14 (43)	6/25 (24)
AML remission lymphocytes	13/47 (28)	0	0/5 (0)

Many studies have also been performed with allogeneic target cells (table 5). Only a low incidence of reactivity was observed against lymphocytes of normal individuals. In contrast, many positive reactions against blast cells were seen; attacking lymphocytes of leukemia patients and also normal controls had cytotoxic effects. Results were also positive with normal control lymphocytes against remission lymphocytes of the leukemia patients.

### Tests With Three Different Assays

As previously reported (2), cellular immunity to antigens on autochthonous blast cells was measured in 20 patients by 3 different assays: skin tests, LCA, and MLC. The results of these tests are summarized in table 6. As noted above, results of skin tests correlated with clinical state. The *in vitro* assays did not

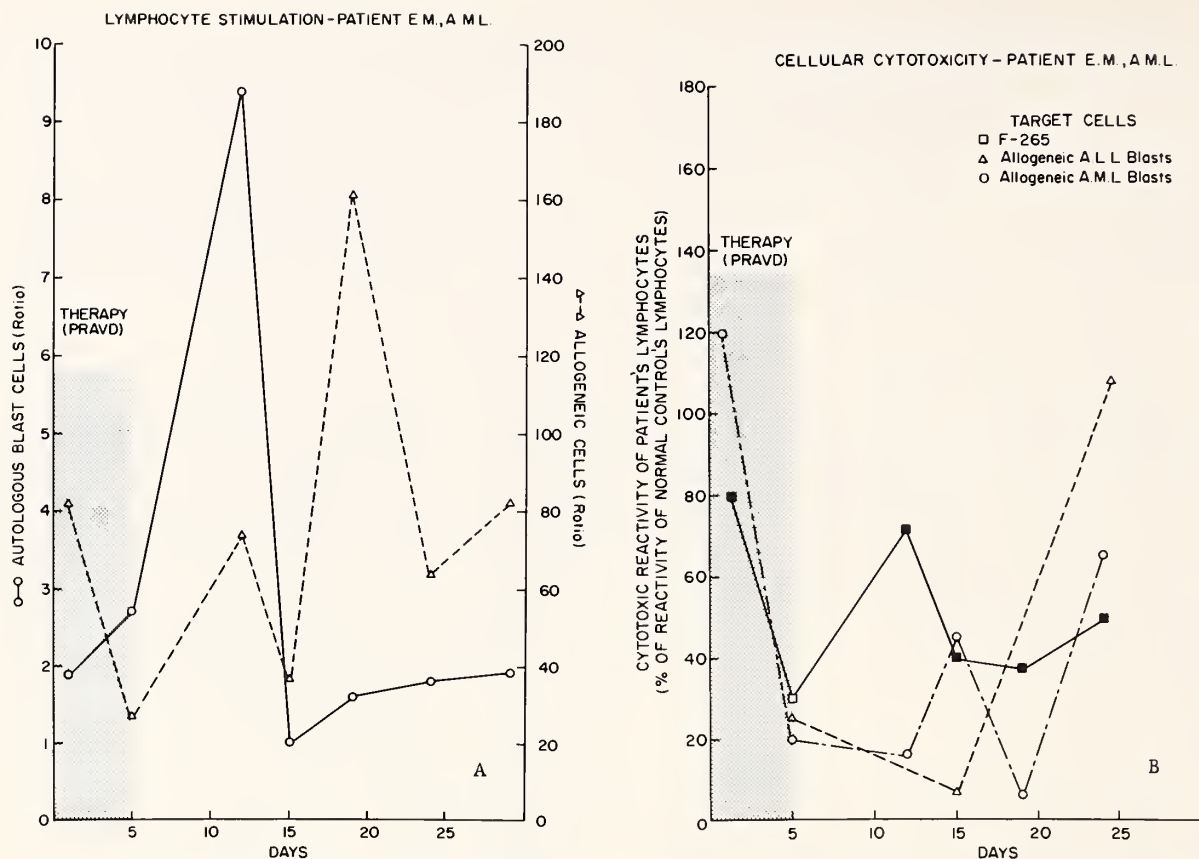
TABLE 6.—Results of 3 assays of cellular immune reactivity in acute leukemia to autochthonous blast cells

Patients	Tests positive/total No. of tests		
	Skin tests	LCA	MLC
ALL, relapse	1/3	1/2	2/3
ALL, remission	6/9	4/10	6/9
AML, relapse	2/5	2/3	3/6
AML, remission	8/9	2/10	9/14
Total	17/26 (65%)	9/25 (36%)	20/32 (63%)

correlate with stage of disease, with as many tests positive for patients in relapse as for those in remission. In addition, when 2 or more of the tests were performed during the same month, the results did not correlate with each other. Skin tests and LCA had the same result 6 times and a different result 6 times. Skin tests and MLC had the same result 7 times and a different result 9 times. MLC and LCA had the same result 6 times and a different result 6 times.

### Effect of Chemotherapy on Cellular Immunity

Positive reactions in MLC to autochthonous blast cells were correlated with the length of time after cessation of a course of chemotherapy (2). All 7 of the tests performed with patients 16–20 days after therapy and 5 of 6 tests performed 11–15 days after therapy gave positive results. Studies closer to, or further away from, time of therapy revealed



TEXT-FIGURE 3.—Effect of chemotherapy (combination of prednisone, cytosine arabinoside, vincristine, and daunorubicin) on reactivity of AML patient in MLC and LCA. *A*: tests in MLC, at various times during and after therapy, with autochthonous blast cells and with the same allogeneic cells. *B*: tests in LCA against allogeneic blast cells and against the human lymphoid cell line, F-265. Results are expressed as the percent of reactivity of patient compared to that of the normal control. Closed symbols indicate significantly positive results ( $P < 0.05$ ), and open symbols indicate results which were not significant.

a lower incidence of reactivity. The results of skin tests or of LCA did not appear to be correlated with chemotherapy in the same way; however, an insufficient number of tests were performed at the appropriate periods (2).

To further explore these observations, serial tests by MLC and LCA were performed on some patients at different times in relation to chemotherapy. The results in 2 are shown in text-figures 2 and 3. Both patients were being treated with monthly 5-day courses of chemotherapy. In both patients, autochthonous blast cells produced maximum stimulation at 10–20 days. Some of the earlier and later reactions were negative; *i.e.*, they had a ratio of  $< 2$ . A phasic response was also observed to allogeneic cells, though the peak in both patients occurred later.

The patients were tested in LCA against 2 allogeneic blast target cells. They were also tested against a human lymphoid cell line, F-265. We have found that the lymphocytes of most normal individuals and patients have cytotoxic activity against this and other lymphoid cell lines (unpublished observations). Since patients with immune-deficiency diseases and patients on immunosuppressive therapy have lower reactivity or none, this appears to be a useful measure of an individual's ability to produce lymphocyte-mediated cytotoxicity. To control for variability in the assay from one day to another, the same normal control was also tested against the target cells. Differences in results against all 3 target cells were seen at the various time points. However, no consistent pattern was seen among different pa-



tients. Patient J. K. (text-fig. 2B) lost significant reactivity to the allogeneic blasts on day 19. Two other patients had increased cytotoxicity at days 15–20. The pattern of J. K.'s reactivity to the blasts was also different from that seen with F-265. Patient E. M. had low results or negative results on each day (text-fig. 3B). She also did not have any cytotoxic activity against autochthonous blast cells (not shown). This was in striking contrast to the high reactivity to autochthonous blasts in MLC.

## DISCUSSION

We have shown here and in previous studies (1, 2, 7, 8) that patients with acute leukemia have cell-mediated immune reactions to leukemia-associated antigens. However, the various assays did not give similar results. The reasons for this poor correlation are not clear. However, several important issues need to be considered in regard to these findings: 1) What is the specificity of each of the tests? Are they detecting the same or different antigens? 2) What phase of the immune response is being measured by each assay? 3) What is the relationship of each test to the clinical state of the patient and his resistance against the tumor cells? 4) Which of these assays, if any, will be useful in monitoring the effects of immunotherapeutic manipulations?

The skin tests appear to be detecting antigens closely related to the leukemic process. In the tests with autochthonous extracts, only blast cell preparations gave positive results. The allogeneic extracts also gave specific results. The reactivity to allogeneic blast antigens indicates that human acute leukemia cells probably have some common antigens. However, the lower incidence of reactivity to the allogeneic extracts indicates that there may be several antigens, each incompletely shared by leukemia cells. Further analysis will be needed to resolve this question. Although we were initially concerned about the possible role of HL-A antigens in the allogeneic skin tests, these antigens do not appear to be important. At the protein concentration (1 mg/ml) used in these studies, no extracts of normal cells produced positive reactions. Yet, assays for HL-A antigens have shown that the normal cell extracts contained at least as much HL-A antigen/mg protein as the tumor extracts (1). It remains to be determined why the HL-A antigens did not elicit skin reactions, particularly in those

leukemia patients who had received multiple transfusions.

The LCA also appears to be detecting leukemia-associated antigens, but the specificity of the reactions may be different from that of the skin tests. In the studies with autochthonous cells, antigens were detected only on the blast cells. In the allogeneic tests, however, results were also positive with remission cells, but not with target cells from normal persons. Most likely the remission cells contain antigens different from those on blast cells, and these antigens on remission cells are undetectable by skin tests. An inhibition assay has recently been developed which should allow more precise determination of the specificity of the antigens detected in LCA (17). The finding of allogeneic reactivity of normal individuals against leukemia-associated antigens is of considerable interest. One possible explanation for these findings is exposure to an environmental agent, such as a virus, with subsequent sensitization against virus-related antigens. The presence of leukemia-associated antigens on remission cells of leukemia patients is also consistent with a viral hypothesis. Morphologically normal cells of mice infected with Gross leukemia virus have been shown to contain tumor-associated antigens (18).

The specificity of the antigens detected in MLC was examined by incubations of bone marrow cells from patients in remission and from patients in relapse (2). In 4 experiments, stimulation was produced only by the "relapse" marrows that contained leukemic blast cells. In 3 experiments, however, remission cells produced some stimulation; in one of these, the remission cells stimulated more than the leukemia blasts. It remains to be determined whether MLC is detecting a differentiation antigen present on normal blast cells as well as on leukemic blasts, and whether the same antigens are being detected in the other assays.

In addition to possible differences in specificity, assays may also be measuring different phases of the immune response [see (2)]. The divergent effects of chemotherapy on results in MLC and LCA are consistent with this possibility. It has been suggested that the various subpopulations of lymphocytes have differential susceptibility to chemotherapy (19, 20).

The relationship of each test to *in vivo* resistance against tumor and to the amount of tumor present needs to be more clearly defined. In the present

studies, only the skin tests for delayed hypersensitivity correlated with the clinical state of the patients. In most patients, the skin tests may provide an indication of tumor burden and give negative results in the presence of large numbers of leukemia cells. However, this correlation has not been complete. Several AML patients and one ALL patient had skin reactivity during clinical relapse. It is very important to determine whether the skin test, or possibly one of the other assays for cell-mediated immunity, can be useful in monitoring the effects of immunotherapeutic measures.

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## Muddying the Water: A Chairman's Prerogative<sup>1</sup>

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I SHOULD like to begin my hopefully argumentative and contentious remarks by resurrecting as questions points which most of us take as valid premises, if not fact. My goal is simply to generate, from the point of view of a devil's advocate, some logical arguments and questions about 3 issues which are relevant, I believe, in that we accept certain basic assumptions about them and we derive our hunches and design our experiments from them. My intent is clearly not to destroy the bases on which we stand, but simply to do some "reality testing" by re-examining our assumptions in the light of recent developments in cellular immunology. Are these assumptions still true? Do they require further experimentation for validation? Do the existing data require alternative interpretations?

The first question concerns the nature of the effector cell in tumor immunity. For some time it has seemed clear that the key to cell-mediated immune responses is the T-cell. We have recently learned that about 30% of peripheral blood lymphocytes are B-cells and, further, that these B-cells can destroy a variety of target cells in the presence of antibodies. Disturbingly, while immune B-cells are capable of specific cytotoxicity, merely coating target cells with an antibody will permit cytotoxicity to be exerted by nonimmune B-cells. B-cells are virtually defined as possessing immunoglobulins at their surface, and Wigzell and his colleagues (1) have elegantly shown that B-cells can be selectively removed from lymphocyte populations by passage through anti-immunoglobulin columns. Under these conditions, the T-lymphocytes are not removed, either because they have no or insufficient quantities of exposed immunoglobulins on their surface. Also pertinent to this discussion are the results of Bianco and Nussenzweig

(2) indicating that B-cells have Fc and complement receptors and MacLennan and Harding (3), that they bind antigen-antibody complexes made with or without complement. Their cytotoxicity is highly sensitive to inhibition by small amounts of antigen-antibody complexes. And lastly, as Baldwin's important observations (4) indicate, generally B-cell cytotoxicity can probably be blocked by haptenic determinants or soluble antigens.

In contrast to recent information about the nature of B-cell cytotoxicity is the classic literature on the role of the thymus-derived lymphocyte in delayed-type hypersensitivity and cell-mediated immune reactions. In fact, most of us take as one of our basic assumptions that T-cells are the effector cells in delayed hypersensitivity *in vivo*. On what evidence is this assumption based? In the early experiments of Miller and Osoba (5) and Good and his colleagues (6), thymectomized animals simply did not reject grafts across major transplantation barriers. Silverstein *et al.* (7) studied the development of graft rejection *in utero* in the developing fetal lamb. They found that the ability to reject grafts occurred in the third trimester of pregnancy, and when it appeared, it occurred with the same speed and intensity as in the adult animal. Thus in development, the appearance of cell-mediated immunity is an all-or-none phenomenon. More relevant is the observation that the ability to reject skin grafts *in utero* appeared weeks before any circulating immunoglobulins were detectable, and proceeded normally even in the presence of excess amounts of heterologous, circulating, anti-immunoglobulin sera. These results again confirmed that grafts could be rejected normally in the absence of immunoglobulins and presumably functional B-cell development. It is well known also that allograft rejection and other cell-mediated responses occur essentially normally in complement-deficient mice, rabbits, and humans, blocked at different stages in the complement sequence. From a variety of studies of patients with agammaglobulinemia, the

<sup>1</sup> Presented at Conference on Immunology of Carcinogenesis, held by the Oak Ridge National Laboratory at Gatlinburg, Tenn., May 8-11, 1972.

<sup>2</sup> Recipient of Public Health Service Research Career Development Award 5K03-19996.



ability to develop delayed-hypersensitivity reactions and graft rejection appears to be quite normal. Although some results conflict on the question, probably T-cell cytotoxicity is not easily inhibited either by anti-immunoglobulin sera or soluble antigens. Most directly, in a series of elegant experiments of allograft rejection in mice, Cerottini *et al.* (8) demonstrated *in vitro* that essentially 100% of the cytotoxic lymphocytes obtained 2 weeks after immunization were T-cells, and could be deleted by treatment with antitheta serum. Is it seriously possible then to question the absolute necessity for T-cells as the cytotoxic effector cell *in vivo*? The answer must be clearly yes, if only because we now know that, for many antigens, the development of immunocompetent B-cells requires the presence of helper T-cells which cooperate with B-cells. If one looks at the now classic experiments, obviously in most cases thymectomy would also produce a marked deficiency in the competence of antibody-producing B-cells. Is there any experimental model *in vivo* from which one can conclude either 1) that immunocompetent B-cells are necessary or sufficient for allograft or tumor rejection *in vivo*, or 2) that they can be excluded as having no function *in vivo* in the rejection process?

Correlative with the distinct capabilities of B- and T-cells are questions of the specificity of these cells and of the antigenic determinants presently being assayed by the various tests *in vivo* and *in vitro*. It is generally accepted that, when individuals are immunized with hapten protein in conjugates, the antibodies produced, and thus B-cells, are "hapten specific," while the T-cells are "carrier specific." When one studies the effects of various antigens on lymphocytes, can one be sure that biologic reactivity to all antigenic determinants is equivalent? Do B- and T-cells see the same determinants? The rejection *in vivo* of an enormous number of chemically induced tumors has been found to be individual tumor specific, and yet the entire premise for detecting cytotoxic lymphocytes in human cancer patients is that they will recognize antigens on tumors of the same histologic type as cross-reacting; *i.e.*, kill is tumor type specific rather than individual tumor specific. Are these cross-reacting antigens presently detected *in vitro* truly tumor-specific transplantation antigens? Does cytotoxicity seen *in vitro* across these cross-reacting antigens parallel cytotoxicity *in vivo*? Is the latter due to all or only some determinants? That this

may be more than a trivial point is suggested by recent work on fetal antigens, mentioned here by Baldwin (4) and seen in other laboratory situations, in which transformation by neoplastic viruses or carcinogens can "expose" fetal antigens on tumor cells. In the case presented by Baldwin and possibly in other studies, many of these antigens gave rise to *in vitro* cross-reactive antibodies, but the rejection of the tumors *in vivo* remained individual specific and apparently did not reflect the cross-reactive immune reaction studied *in vitro*. Similarly, Morton *et al.* (9) many years ago demonstrated that, while mammary tumors bear a strong, common, cross-reactive antigen, animals rendered tolerant of the mammary tumor virus antigens also demonstrated individual-specific tumor immunity, just as seen with chemically induced tumors. Which of these is more important in the rejection process, or are they simply equally "immunodominant"? In addition to knowing what cells are involved in killing *in vivo* then, we must also question what antigens each of these cells is reacting to, to know which are crucial to the rejection process.

The last major thesis I believe we are beginning to take for granted is blocking. Clearly most tumors in experimental animals that have been studied are antigenic. The phenomenon of concomitant immunity proves *in vivo* that individuals with cancer can or may have immune responses to their tumors. Then why do they not reject their cancers? From a series of extraordinarily imaginative *in vitro* experiments, the Hellströms (10) hypothesized that, at the same time that individuals may possess cell-mediated immunity against their tumors, they may have circulating factors blocking the cytotoxic effect of lymphocytes *in vivo* and *in vitro*. In essence, this hypothesis greatly unifies and simplifies a variety of diverse experimental and clinical observations by emphasizing the regulation of the cell-mediated "surveillance" function by humoral responses and antigen-antibody reactions. Like any good hypothesis, it is experimentally testable, and, as we have heard, a great deal of evidence has been adduced to support it. And yet the blocking hypothesis raises many questions: 1) It has been clearly demonstrated *in vitro* that target cells coated by antibody or antigen-antibody complexes can be killed by B-lymphocytes, even nonsensitive ones. Why does coating target cells with blocking antibodies or serum lead to blocking and not enhanced kill by the B-lymphocytes which

have complement receptors and Fc receptors? Even in a highly sensitized individual, one would hardly expect more than 2% of the lymphocytes at the most to be sensitized to any given antigen. Thus while the antibody or antibody-antigen complexes may block that 2% from exerting specific cytotoxicity, it is difficult to understand why it does not permit the 98% normal lymphocytes to kill the target cells coated with antibody or antibody-antigen complex. 2) Where is the immunologic battle against cancer fought? Is it fought locally at the tumor site or in the regional nodes or is it fought systemically? If blocking is a major factor leading to dissemination of cancer, then do the pathologists have evidence for plasma cell proliferation which might produce the blocking factors, *a*) locally near the site of primary or metastatic tumors, or *b*) in the regional nodes, to permit escape from immune destruction and thus metastasis, or *c*) systemically throughout the lymphoid system to provide a large enough pool of circulating complexes or blocking antibody to permit metastases to develop with impunity anywhere? 3) If the blocking factors are in part antibody-antigen complexes, one would predict that the pathologists would find, especially in patients with advanced cancers, deposition of immune complexes in the kidneys. This is a simple prediction of the model—has it been tested? 4) Can the blocking hypothesis account for tumorigenesis, or does blocking merely occur after large tumor masses develop? With a small tumor, one might expect only very small amounts of soluble tumor antigens to be released—perhaps too small to lead to a systemic antibody response and hence not of much assistance to the tumor in blocking the cellular response. Conversely, if blocking complexes occur only with larger tumors, we remain at a loss to explain immunologically why some tumors develop and why others are rejected. 5) The phenomenon of concomitant immunity mentioned above also is difficult to understand in terms of circulating blocking factors. How can one explain the fact that the small, transplanted biopsy is rejected at another site in the same individual while the primary tumor grows progressively to kill the animal?

It is easy to raise these and other questions about our assumptions regarding the nature of tumor immunity; it is difficult to generate definitive answers experimentally. Only recently has it been possible to examine, *e.g.*, as Perlmann *et al.* (17)

are doing, *in vitro* techniques that are being used to answer these questions in terms of which cells in man are destroying tumor cells—B-lymphocytes, T-lymphocytes, or both? This question in turn has lead to re-asking the question of which cell plays the decisive role in destruction of tumors *in vivo*. In my view, this is a fundamental gap in our knowledge which can soon be answered. If there prove to be differences in the effects of B- and T-cells, then it becomes of importance to define antigens recognized by both cell populations and by cell-mediated immunity versus immunity antibodies. Which antigenic determinants are involved in the destruction of tumors, which are not, and which engender enhancement? Answers to all of these questions are crucial to evaluation of the blocking hypothesis. If B-cell cytotoxicity is of real importance *in vivo*, clearly these cells can be easily inhibited by antigen-antibody complexes or free antigen, and the observation of B-cell killing and blocking in *in vitro* systems becomes a highly relevant index of reality *in vivo*. If, in contrast, tumor immunity *in vivo* is primarily a T-cell function, then much of what we have all been preoccupied with in *in vitro* assays may really involve B-cell artifacts. If B-cells producing antibodies are cytotoxic *in vitro*, it is not terribly surprising that they can regularly be inhibited by soluble complexes as well, so that we may be simply measuring *in vitro* killing by B-cells and its inhibition by their own gene product, antibody, without disclosing anything about tumor immunity *in vivo*. Undoubtedly our panelists and members of the audience have thought more deeply about many of these questions than I. I hope that I shall learn that many of these questions have either been answered or are simply not so important after all.

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## GENERAL DISCUSSION

**B. R. Bloom:** Dr. Perlmann, what is the evidence *in vivo* that B-cells may be involved in tumor immunity?

**P. Perlmann:** First, I have been careful to avoid the expression "B-cell killing." I was talking about T-independent lymphoid cells. Care is required because, by definition, a B-cell is the precursor cell of an antibody-forming cell, and we have no good evidence that these cells are involved in any of these antibody-induced killings.

The essential point you raise, of course, is: What evidence do we have that antibody-induced killing by lymphoid cells plays a role *in vivo*? I cannot say very much on this question. There are now, to my knowledge, a number of experimental models in animals in which one tries to correlate tumor progression with the development of cytotoxic lymphocytes and with the development of antibodies which induce cell-mediated killing. There is evidence in the Moloney sarcoma viral system—being studied by Drs. E. Klein and E. Lemon in Stockholm—that the latter mechanism may be of importance for surveillance under later phases of tumor growth.

I would like to make another point. In those *in vitro* studies in which one looks for blocking or killing inducing reactivity of antibodies in the sera, it has been noted that cytotoxic reactivity of the lymphocytes at a certain dose level may potentiate the cytotoxic reactivity at a higher dose level and, when one increases dose even further, one may again get a strong inhibition.

**J. Stjernswärd:** As the Chairman's question referred specifically to *in vivo* systems, I will respond. There are very few data and direct *in vivo* analyses in the cancer patient. But some data which might give the answer are the findings in irradiated patients in whom—if we think our analyses of B- and T-cells are relevant—we diagnose a lymphopenia which we know will be of a magnitude that will allow a homograft to be prolonged. We find a shift, an increase of B-cells and a decrease of T-lymphocytes proportionally, in a group of patients, many of whom have "minimal residual tumor cells" evidenced by later occurring metastases.

So these data may give some answers on whether observed changes in immune status, especially B- and T-cells, correlate with earlier metastases. That would be a nice model. You also alluded to blocking antibodies. We know that, if you irradiate a patient, he may get increased auto-antibodies in the organ irradiated. This may be only a quantitative increase, not always a qualitative increase, due to a relative increase of B-lymphocytes. It may be that blocking antibodies or Ag-AB complexes also will increase. We may get an increase of B-cells producing blocking antibodies and a decrease of T-cells. From final results of the few critical, well-documented, randomized studies available in the world, we find increased early metastases.

I have another point to make. One must be cautious in considering findings found in artificial *in vitro* systems as valid and clearly representative of the complex immunity *in vivo*. I doubt whether immunity found against autochthonous cancer is comparable and mediated in the same way as that found against xenogeneic or even allogeneic

target cells. As pointed out earlier, erythrocytes used by many experimentalists are much easier to destroy than, *e.g.*, tumor cells. The additive immunogenic effect of allogeneic HL-A antigen when allogeneic tumor cells are used, represents another unknown, *e.g.*, by causing nonspecific stimulation or committing bystander cells. The immune response toward weak antigens like the non-H-2, and probably most tumor-specific antigens, seems to differ basically from that toward stronger antigens like the H-2. Against the first antigens, it is easy to induce tolerance and enhancement and more difficult to prepare antibodies, and a lower number of lymphocytes seem to respond to them. Against the stronger antigens, it is more difficult to induce tolerance and easier to prepare antibodies, and a higher number of lymphocytes seem to respond primarily to them. By immunization, proportionally more lymphocytes are committed toward weaker antigens than stronger. However, all this was analyzed by our Chairman; but I was motivated to make this comment because mechanisms explored in allogeneic and even xenogeneic model systems *in vitro* are being discussed as representative for possible tumor-specific immune mechanisms *in vivo*. The seductive and habit-forming cocktail, which is based on a super-elegant technique being mixed equally with a xenogeneic system—but not even spiced with a tumor—should be taken very carefully and not daily. Whenever tumor immunity is analyzed, it should be done in autochthonous or syngeneic systems.

**Bloom:** I would like to add one thing in answer to the question of what we know about B-cells *in vivo*. The old studies that I dragged up, of necessity, fail to take into account a very obvious point, in light of our modern knowledge, about antibody formation. When one takes the thymus out of an animal, observes that the animal can't reject a graft, and remarks "That's T-cell mediated,"—that is how those experiments have always been done—one fails to consider the existence of a B-cell response which is thymus dependent. Those animals would not have made antibodies to sheep red blood cells well either, since T-cells are required for cell cooperation in many B-cell responses.

So, what I'd like to bring out in the question is a point that Dr. Perlmann stated very well. We don't really know, in *in vivo* situations, whether the B-cells are in fact not involved. What we do know is that, clearly, there are situations in which, if B-cells were involved, they would require T-cells to function.

I wonder if Dr. Cerottini, who has studied the classical cytotoxic system in the mouse, would like to comment?

**J. E. Cerottini:** As already mentioned by Dr. Perlmann, evidence is ample that killer cells involved in allogeneic systems are T-cells. The experiments which led to this conclusion have been reviewed recently.<sup>1</sup>

The best evidence, can be obtained in the mouse because specific antisera to T- and B-cell markers are available. To summarize, if one takes an immune spleen cell population

<sup>1</sup> BRUNNER KT, CEROTTINI JC: Progress in Immunology. New York, Academic Press Inc., 1971, 385 pp.

containing both cytotoxic lymphocytes and alloantibody-forming cells, it's very easy to demonstrate that treatment with antiserum to T-cells (anti-theta or anti-MSLA) and complement abrogates the cytotoxic activity, whereas the alloantibody, plaque-forming cell (PFC) activity remains. Conversely, after treatment with an anti-MBLA serum, which is an anti-B lymphocyte, and complement, cytotoxicity is still present, whereas alloantibody plaque formation is eliminated.

The main question is: Are these cytotoxic lymphocytes doing anything *in vivo*?

As Dr. Bloom mentioned, all the experiments tending to demonstrate that cell-mediated immunity is important in tumor rejection have used mixed cell populations, containing both immune T- and B-cells. We have repeated these classical experiments using purified populations of T- or B-cells. We found that allogenic tumor grafts could be completely rejected in heavily irradiated animals given injections of a pure population of immune T-cells. Thus, in this system, it's quite clear that the effector cells are the immune T-cells and macrophages are not required.

If this is valid for this system, does it mean that it applies to all systems? Further studies are needed to elucidate this question. Using procedures similar to ours, Dr. Tevethia found that, in a syngeneic tumor system, the immune T-lymphocytes require something else to inhibit tumor growth.

**Bloom:** First let us turn to the question of blocking: How do "blocking factors" cause tumor growth if there is only a small antigen load? Also, how can one explain concomitant immunity by circulating "blocking factors"?

**K. E. Hellström:** You asked how the blocking phenomenon could be so important, if only small amounts of antigen are released from the tumor as it starts growing. To the extent this has been studied, we have been struck by the fact that blocking serum factors appear early,<sup>2</sup> at about the same time point we first can detect specifically cytotoxic lymphocytes. Whether the local concentration of blocking factors is higher around the tumor than in the serum is not completely clear, but the fact that one can elute blocking factors from tumors growing *in vivo*<sup>3</sup> indicates that the amount of blocking factors may be higher in the area of the tumor than elsewhere in the organism and that lymphocytes meeting growing tumor cells may be blocked also when the level of serum blocking activity is rather low.

You also asked how one can understand the phenomenon of concomitant tumor immunity, if the blocking serum activity plays the great role we have said. I do not find this difficult at all. In Kaliss' classic studies on the enhancement of allogenic tumors, he found that an animal carrying an enhanced tumor graft often rejected a second transplant of

the same tumor. The simplest interpretation of this phenomenon (as of the concomitant immunity to a syngeneic tumor in an animal carrying an already growing one) is that the local concentration of blocking factors is higher around the growing tumor (where both tumor antigens and antibodies to them are more abundant) and, also, that the freely suspended cells of a new implant are more vulnerable to the circulating immune lymphocytes than are the tumor cells growing together within a lump. One must also remember, of course, that the presence of blocking serum factors in tumor bearers does not imply that *all* cell-mediated immunity is blocked; if the blocking is only partial, concomitant immunity would be expected, with the original tumor, surrounded by elutable blocking factors, continuing to grow.

Another point: Someone wondered why one cannot elute blocking factors from the kidneys. I believe kidneys are, indeed, good places to look for blocking factors, since complexes between antigens and antibodies are likely to settle there. In Sjögren's experiments, which you heard about, however, the kidneys were washed several times before the elution, and it is likely that complexes not firmly bound to tissue structures, such as tumor antigens at the surface of neoplastic cells, were then washed away.

**W. J. Martin:** The generalization that antigen-antibody complexes are blocking and antibody alone is not blocking, and perhaps even deblocking, may not always be valid. Thus, it has been reported that blocking factor is present in the sera of multiparous mice previously pregnant to allogenic males. Even though the animals are no longer pregnant, they still have blocking material in their sera. It is perhaps difficult to envision circulating antigen in these animals and suggest that antibody alone can block. Again, with hyperimmune sera capable of passively enhancing tumor growth, one would expect these sera to contain antibody rather than antigen-antibody complexes. Another issue is that, if antigen is present in blocking material, then I wonder whether, in Dr. Sjögren's experiments, the antigen contained in the antigen-antibody complex may immunize the recipient in a way less efficient than would be obtained with live cells. Thus the greater tumor growth, which he attributes to transfer of blocking per se, may in fact be due to the development of a weaker cell-mediated immunologic response.

**K. E. Hellström:** I do not think I made any generalizations that antibody alone can never block (as a matter of fact, I stated it could but that the blocking seen with sera from tumor bearers is more likely mediated by antigen-antibody complexes than by antibody alone), and I have said nothing as to whether the blocking seen with sera from pregnant animals is due to antigen-antibody complexes or to antibodies, since we have not studied that. However, it could be very possible that the pregnant mice do, indeed, have circulating antigens from the fetus together with antibodies and that these stay around a short time after completion of the pregnancy. I do not think one can use the pregnancy situation as an argument as to whether or not the blocking one sees with serum from a tumor bearer is due to antigen-antibody complexes, to antigen alone, or to antibody alone.

I firmly believe that the enhancing hyperimmune sera

<sup>2</sup> HELLSTRÖM I, HELLSTRÖM KE: Colony inhibition studies on blocking and non-blocking serum effects on cellular immunity to Moloney sarcomas. *Int J Cancer* 5:195-201, 1970. HELLSTRÖM I, HELLSTRÖM KE, SJÖGREN HO: Serum mediated inhibition of cellular immunity to methylcholanthrene induced murine sarcomas. *Cell Immunol* 1:18-30, 1970.

<sup>3</sup> BANSAL SC, HARGREAVES R, SJÖGREN HO: Facilitation of polyoma tumor growth in rats by blocking sera and tumor eluate. *Int J Cancer* 9:97-108, 1972.



studied, *e.g.*, by Kaliss, contain antibodies, and I doubt they contain much antigen, if any. However, the cellular mechanisms can, of course, be the same as postulated by us for the syngeneic tumor enhancement (although they do not have to be!), since the hyperimmune sera may bind to the antigens of some transplanted tumor cells and release them, thus forming antigen-antibody complexes which can act on the lymphocytes. As far as Dr. Sjögren's experiments are concerned, he should comment on them himself.

**H. Sjögren:** We have tested the lymphocytes' cytotoxicity in the recipients of the blocking and the control material at 2 time points, days 4 and 8 after inoculation. The blood lymphocyte activity was quantitated by dilution of lymphocytes to a point where they were no longer active. The recipients of blocking material had the same level of cell-mediated immunity as the controls measured this way. This indicates that it is not an afferent type of blocking in this system.

**R. W. Baldwin:** I would like to reply to Dr. Martin and to emphasize what Dr. Hellström said. In the rat hepatoma system, we can show that tumor-bearer serum blocks cell-mediated tumor killing and certainly one can make antigen-antibody complexes to be blocking (Baldwin, Price, and Robbins, *Nature* (Lond); (in press). Sera from rats hyperimmunized against rat hepatomas contain tumor-specific antibody demonstrable by membrane immunofluorescence, and these sera show complement-dependent cytotoxicity for plated hepatoma cells. But, without complement, these sera are blocking; therefore, one can get antibody to act in a blocking capacity, and I think we are talking about 2 different situations here in comparing tumor bearers and immunized hosts.

**J. D. Feldman:** I would like to direct a question to Dr. Sjögren and Dr. Hellström. Does either of you have any direct evidence that antigen or antigens are involved in an antigen-antibody complex to cause these blocking effects?

Also, I think that, when you are talking about blocking, you must distinguish between blocking at the target site and blocking at the effector-lymphocyte level. In the former, you would only have to postulate antibody. In the latter, you must postulate something more than antibody, *e.g.*, antigen-antibody complexes. Further, one has to not only distinguish between these 2 loci but also separate them temporally.

**Sjögren:** I want to comment on the evidence for antigen in the form of antigen-antibody complexes being involved in the blocking, as it is routinely assayed *in vitro*. The evidence we have is indirect, and full proof is not yet available. The blocking activity in a serum can be adsorbed specifically on tissue-cultured tumor cells of the type in question. It can then be eluted at low pH. From the eluate we can separate—by Amicon ultrafiltration at maintained low pH—one fraction containing molecules above an approximate molecular weight of 100,000 (which would contain the immunoglobulins, of course) and another fraction containing molecules between molecular weights of 10,000 and 100,000. We call these fractions E100 and E10, respectively. None of these fractions is blocking *in vitro* by itself, but when the 2 fractions are mixed at neutral pH, the blocking is restored.

Fractions E100 and E10 have been produced in 2 tumor

systems that do not cross-react. When E100 of one tumor type is mixed with E10 of the other, blocking is not restored in any of the systems. Thus the specificity of blocking is determined not only by the E100, or the immunoglobulin, but also by the low-molecular-weight fraction. We believe that the simplest explanation for this specificity is that the fraction E10 contains the tumor antigen.

**R. B. Herberman:** I would like to bring up 2 points. First, in terms of the possible role of blocking, one of the points that I've been a bit concerned about with the MSV system is the time of appearance of blocking factors in relation to tumor growth. Dr. Sjögren and others have nicely shown that, early after injection of the virus, there are blocking factors at the time the tumor is getting larger, but generally, about 2 or 3 days later, the tumor starts getting smaller. This raises some questions about what causal relationship these blocking factors really have to progressive growth of the tumor, since the tumors can regress so rapidly thereafter. I wonder whether some of the phenomena that we are measuring are simply related to the temporal sequence of the immune response, as Dr. Feldman just suggested.

With a Gross virus-induced leukemia in rats, we observed that, if one measures short-term chromium-51-release cytotoxicity by cells, there is a peak cellular response at about 10 days, and then beyond 20 days the activity becomes undetectable in the immediate assay. However, if one takes cells from the animals that are immediately not cytotoxic and incubates these cells by themselves for over 12 hours *in vitro*, as Dr. M. Ortiz-Landazuri, who is in our laboratory, has demonstrated, the cells become quite cytotoxic against the target cells. This would point away from an effector target cell block, but toward a central block of the lymphocytes *in vivo*. When the lymphocytes are taken out of the environment of the host for a time, these cells then can become active.

**I. P. Witz:** It's quite easy to conceive how antibody alone and antigen alone would block. For a complex to block both at the target cell level (as shown by the Hellströms in many cases) and at the lymphocyte level (as shown by Sjögren and the Hellströms), one would have to have complexes which have both free antibody-active sites and complexes with free antigenic determinants.

**Bloom:** There is no reason to assume that there is only one molecular complex when you have a gradient of antigen and a gradient of antibody. Why can't you have the whole range of complexes?

**Witz:** I think from what Dr. Sjögren said before that it's one and the same complex.

**Sjögren:** For routine assay of blocking *in vitro*, the blocking factor must be adsorbed to the surface of target tumor cells; *i.e.*, a free combining site on the antibody part of the postulated antigen-antibody complex is essential. I do not think that our results indicate whether this is the only type of complex present in a blocking serum. I would agree with you in saying that there are most likely free antigen or free antibody and complexes of at least 2 kinds in a blocking serum: one type of complex with all the combining sites occupied by soluble antigen and the other type with still one free combining site. Some of the data Dr. Baldwin presented fit very well with this proposition.



You recall that, when Dr. Baldwin added soluble antigen prepared from tumor tissue to nonblocking antitumor sera, the sera became blocking. When antigen was added in excess, the blocking disappeared, possibly due to the formation of complexes without any free combining sites left.

**Perlmann:** I tried to make the point in my presentation that different effector cells may interact in different ways with antigen with antibody or with antigen-antibody complexes. You can inhibit the T-cells by antigen, and there is no reason why this couldn't be due to an antigen-antibody complex which has free antigenic sites to inhibit. On the other hand, when you have an effector cell with an antigen-antibody receptor site, then it is sufficient with the Fc part of the antibody and antigen-antibody complex to block effector functions. This has been shown experimentally.

**G. W. Santos:** This question is directed to Dr. Herberman and anyone else on the panel who cares to answer. Perhaps I object to lumping all tumors together, and my generic question is: What evidence is there that cellular immunity is important in a host response as far as leukemia is concerned?

I ask that question because Dr. Herberman showed some type of correlation with skin testing, but I think it has been several people's experience that, when a patient has a full relapse, he is relatively anergic to skin testing. I hope you won't argue that point. Then, when the patient is in remission, he might respond.

When the patient begins to relapse, does he lose any of his other skin tests? If he does, then the skin testing with tumor loses its specificity.

**Herberman:** I think it's true, as Dr. E. Hersh's laboratory and your group have shown, that leukemia patients who have very florid disease do become less reactive or actually become anergic in skin tests. Most of the tests we have performed were on patients in an early stage of relapse, in whom there would be from 20-50% blasts in their bone marrow but generally no circulating blasts at the time of the testing.

We have not seen a decrease in general reactivity of the patients.

In each of the serial tests we have done, patients who were reactive to mumps, candida, or other recall antigens when they were in remission retained reactivity to each of these at the time that they had their relapse.

**O. Stutman:** I would like to comment on the antigen-antibody complex problem, because at least in the mouse mammary tumor system with which I have been working, we cannot find evidence that the blocking effect is mediated by complexes. The antigen involved is the ML. It's a cell-surface antigen, related to the virus but not present in the virus. It can be solubilized from tumor cells. We can show that both soluble antigen and antigen-antibody complex can stimulate blast formation of lymphocytes. That is our assay for presence of antigen.

We did separation experiments described by Dr. Sjögren and found that all the blocking activity was present only in the high-molecular-weight fractions, but we could never detect antigen in any of them.

So, at least in this particular system, the blocking effect seems to be mediated by immunoglobulins.

On the other hand, we can remove the blocking activity by solid immunoabsorption in columns, containing rabbit antimouse immunoglobulins, but we cannot remove the blocking activity by immunoabsorption with rabbit antibody directed to the ML antigen.

**S. R. Rosenthal:** I wish to present a model wherein the blocking "antibody" may be related to the target cell itself. My group and I have evolved a theory of immune response which differs from the classical one involving cellular or humoral immunity. This theory states that, beginning with a given antigen, if one fragments this molecule by mechanical (sonication or high pressure) or by enzyme action, injects these fragments (competition) in a given host, and then 5-24 hours later injects the whole antigen, the host will fail to react to the complete antigen. For example, burn toxin injected intravenously in a mouse is lethal. If one fragments this toxin by freezing and thawing, plating, and finally sonication (1 hour at 9 kc-150-w input), this competition fails to kill the mouse. If 5-24 hours after the injection of this competition one injects the original burn toxin, the mice are protected. Results were similar with diphtheria toxin in guinea pigs. The mechanism of action is explained as a competition between the fragmented antigen as the receptor site of target cells and the complete antigen; *i.e.*, the partial blocking of the receptor site competes with the action of the original molecule.

The competition phenomena were applied to tumors as follows. Sarcoma 180 was allowed to grow subcutaneously in Swiss mice to attain 1 or 2 cm in diameter. A portion of the tumors was removed and a competition prepared. If the competition was inoculated intravenously 2 or 3 times a week in the tumor-bearing mice, after 2 or 3 weeks there was an enhancement of the tumor size in these animals compared to those given injections of saline. Possibly the competition negated the effect of the blocking agent on the target cells. Interestingly enough, acetone extracts of the competition caused regression of tumors in one-third of the animals; in one-third, the tumors remained stable, and in one-third they were enhanced in size.

**W. G. Hammond:** Rather than get too involved in blocking antibody, and so on, I would like to raise a much more fundamental question. It's pretty clear, from all the data of many studies of circulating tumor cells in patients with cancer, that a lot more cells are shed into the bloodstream—than ever light and form metastases. In the animal laboratory, the corollary is the phenomenon described years ago and still called "concomitant immunity." I think this phenomenon exists both in the laboratory and in patients. I wish to ask Dr. Sjögren, is there any evidence, or are there any experiments, that reconcile concomitant immunity as a phenomenon, which is quite clear, with the blocking factor concept? One can think of many possible ways that it can be resolved, but are there any tangible data which do provide evidence for a resolution? If concomitant immunity is not resolvable with the blocking factor concept, then perhaps the blocking factor concept isn't worth all this much fuss.

**Sjögren:** I don't see why concomitant immunity would be so difficult to reconcile with the blocking factor concept. In addition to the comments already made by Dr. Hell-

ström, I want to point out that blocking *in vivo* is likely to be partial in most cases rather than complete. *In vitro* we often observe such a partial blocking.

If we accept that we have a partial and not a complete blocking, then why are isografted cells more easily rejected than the cells of an established tumor by the remaining unblocked cellular immunity? Possible reasons for this increased susceptibility to the immune elimination are a better exposure to the effector lymphoid cells and other nonimmunologic growth-inhibitory factors.

Concomitant immunity is usually not very strong. For example, it has been demonstrated in several experimental systems that the isograft resistance is clearly detected some time after complete surgical removal of a growing tumor, while it is not readily detectable in the tumor-bearing animals. We have studied this phenomenon in relation to the blocking phenomenon in rats carrying polyoma tumors. Compared to controls, animals isografted within 2 days after the original tumor was excised, when the serum-blocking activity was still detectable, had grafted cells that grew out more rapidly but then grew somewhat slower. The same dose of tumor cells did not grow out at all in rats whose original tumors had been excised 6 days before isografting and which at that time no longer had any serum-blocking activity. These results fit extremely well with the blocking concept. With regard to the tumor-host systems in which a fairly strong concomitant immunity has been reported, I believe that it would be premature to make further specific comments before the blocking activity of such animals has been studied in some detail.

**Bloom:** Does anybody in the audience have experience with data on tumor incidence in agammaglobulinemic patients? Do they have a higher incidence of tumors?

**C. McKhann:** They have a high incidence of tumors.

**Bloom:** And they can't block, because they don't have antibodies.

**H. T. Wepsic:** I have two questions. One is more in the way of a comment to Dr. Wunderlich. In Dr. Wunderlich's system, a lymphocyte can kill at least 1 tumor cell and, maybe, 2 tumor cells. In our experiments, using the animal model *in vivo*, I showed that the lymphocytes would go to an antigenic tumor site and become unavailable to go to a peripheral site and kill that tumor. I called this "abrogation of transfer," but we may have also called it "consumption of the lymphocyte immunity," which was passively transferred. This was quite specific. It was done in 2 different ways with tumor specificity. The abrogation of passively transferred tumor immunity was also related to the dose of tumor cells passively administered.

Also, Dr. Sjögren, were there animals who did not receive any treatment other than splenectomy?

If you do not have such an experimental group, you are going to have considerable difficulty sorting out whether your deblocking antisera are really therapeutic.

**Sjögren:** We know, on the other hand, that in the isograft situation, they are being treated only with the unblocking serum that we could unblock, and we got first an initial outgrowth and progressive growth for 2 weeks of the isograft and then regression on the isograft itself, and this was only by treatment with unblocking serum; on that

basis I would say we have no positive evidence that splenectomy would affect the growth of tumors to any dramatic extent.

**N. Levy:** I would like to ask a few questions and then also relate some data that we have on the fractionation of blocking sera. We eluted 3 blocking sera off diethylaminoethyl-cellulose and found the blocking activity in the IgG fractions. One of these sera likewise eluted from Sephadex G-150 with IgG.

This is our human brain tumor system using a modification of the Hellströms' microcytotoxicity test. Also I would like to ask Dr. Perlmann if he has used his partially purified tumor antigen preparations to induce blocking of cytotoxicity by lymphocytes which have been passed across his anti-IgG-loaded columns.

In addition, getting at this wondrous molecule alluded to by Dr. Witz, I would like to ask Dr. Sjögren if he can absorb the blocking activity, which he presumes is due to antigen-antibody complexes, with lymphocytes specifically immune to the appropriate target cells.

**Perlmann:** We have not yet done this very important experiment.

**Sjögren:** This is an obviously important thing to be done, and we have started such experiments. I am not yet ready to report any results.

**McKhann:** I would like to offer an explanation to the findings of Dr. Stjernswärd and Dr. Herberman. Dr. Stjernswärd showed that the lymph node cells of his patients could not be stimulated *in vitro*, and Dr. Herberman showed a reverse relationship between cellular cytotoxicity and the capacity of the lymphoid cells to be stimulated *in vitro*.

Perhaps what we are seeing here is a progressive change in the population of lymphoid cells (fig. 1). Virginal cells can be stimulated by tumor, *in vitro* or *in vivo*. They undergo blast transformation and one or more divisions. This results in a population of effector cells now capable of attacking tumor cells *in vitro* and presumably *in vivo*. This entails a real shift in the population of lymphoid cells, depleting the compartment of cells capable of being stimulated to undergo blast transformation while building up the population of cells capable of actual cytotoxic attack. The relatively poor regression of regional lymph node cells from tumor-bearing individuals to undergo stimulation *in vitro* probably indicates that they have *already* undergone stimulation *in vivo*. We have evidence in mice that this may be true, because the lymph node cells from normal mice undergo good stimulation upon cocultivation with syngeneic sarcoma cells, while the lymph node cells from tumor-bearing mice do not. Instead, lymph node cells from the tumor-bearing mice have a very high background level of activity in terms of uptake of tritiated thymidine, indicating that they have already been "turned on."

Dr. Stjernswärd, do you have high background activity in the regional lymph nodes of the patients with tumors?

**Stjernswärd:** Yes, that is true. But even if it is corrected for that, the findings are still there. Tumor-draining lymph node cells from 6 of 8 patients were specifically nonreactive against autochthonous cancer. This was not due to nonviability, since they could respond to the stimulus of allogeneic cells and they responded against phytohemag-



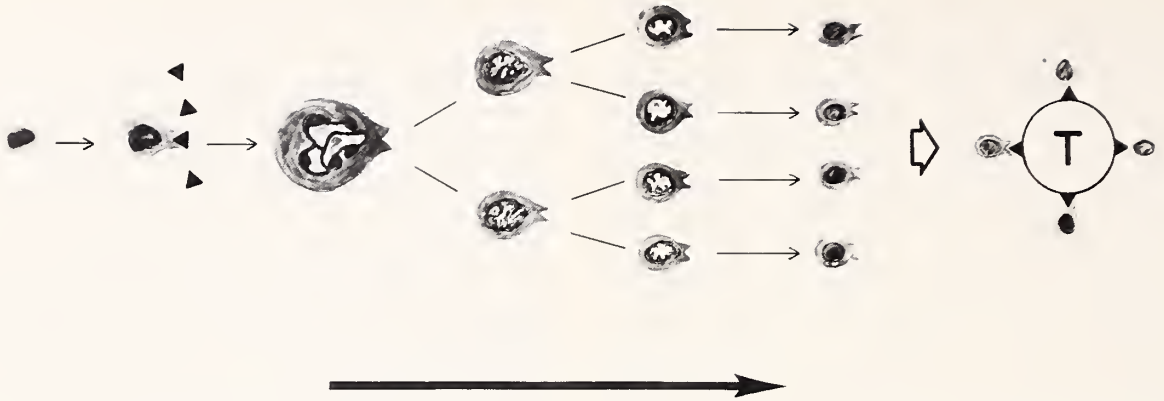


FIGURE 1.—Progression of lymphoid cells from stimulation through the development of specific cytotoxic potential. Nacent lymphoid cell is capable of being stimulated by contact with the appropriate antigen undergoing blast transformation and cell division(s). The end product of this series is the effector cell capable of direct lymphocyte cytotoxicity when brought in contact with the tumor cell. Normal animals have a moderate-sized pool of Nacent cells capable of being stimulated by a specific antigen but no conditioned effector cells. Conversely the tumor-bearing animal has many effector cells but a markedly depleted pool of Nacent lymphoid cells of the same specificity, this pool having been depleted by continuous *in vivo* stimulation.

glutinin to the same degree as the peripheral lymphocytes. It was not due to nonantigenicity of tumor cells, since they stimulated peripheral lymphocytes. Whether the results are due to the fact that tumor-specific anergy has occurred in the lymphoid cells or that the clone of cells able to respond to tumor-specific stimulus already is exhausted or maximally stimulated is too early to say. Do you agree?

**McKhann:** Exactly.

**Stjernswärd:** That would be the point that the figure should bring out.

Regarding the second point raised. It is very dangerous to extrapolate between the stimulation seen against tumor cells from lymphoid system and solid sarcoma. Lymphoid cells as moving circulating cells are made to recognize each other. Signals occurring when doing so may be caught as increased DNA synthesis in our tests. Autochthonous lymphoid cells from different cell compartments may stimulate each other when brought into contact, as indicated by Dr. Herberman's findings where autochthonous bone-marrow cells stimulated peripheral lymphocytes in the mixed lymphocyte culture. We have found that a few days' culture of peripheral lymphocytes *in vitro* may stimulate fresh peripheral lymphocytes sampled later to increase DNA synthesis upon contact in the mixed lymphocyte culture test. I am less secure about interpreting the stimulation of the peripheral circulating lymphocytes by local lymph nodes: It may represent stimulation by tumor product, *e.g.*, tumor antigen, or possibly lymphoid cells from different cell compartments are brought into contact and the stimulation found represents a normal recognition and contact signal.

**M. M. Black:** I have a feeling of *Alice in Wonderland*: "It gets curiouser and curiouser!"

I hear every once in a while the word "realities" by some speaker, and then I get lost in a jumble of very specific, precise, and very abstract techniques that have nothing to do with tumors, as I see it.

Now I remember about 20 years ago, when I was one of the few voices that said: "There are such phenomena as immunologic reactions operating in the tumors." I used to tell my students, or pathology audience, when they talked about the importance of grading tumors: "Around every tumor there is a patient, and an important part of this patient is the lymphoreticuloendothelial system, and it operates in a tumor-host interaction."

Now I find it necessary to say: "In addition to the lymphoreticuloendothelial system, there is a tumor." It is also variable in potential. Patients having breast cancers with highly differentiated nuclei will have excellent survival, regardless of what the surgeon does or what the lymph nodes do.

Conversely, patients having breast cancers with undifferentiated nuclei will die very rapidly.

So there is a variable in the tumor and in the lymphoreticuloendothelial system; moreover, there may be even a greater variable, having nothing to do with the reticuloendothelial system or the tumor.

The point was mentioned before about concomitant immunity and tumor cells circulating in the blood. Of course, that was the original study on concomitant immunity. I don't believe that we should think that we have final answers in terms of immunologic information alone. At the same time the tumor is metastasizing to the bone in a particular patient, it may or may not be metastasizing to the liver; or at the same time metastases in several areas of the same organ may be growing differently.

The other point in general is that we set up model systems concerned with local growth. Most cancer patients do not die of the local growth. They die of distant metastases, which may or may not correlate with the size of the local growth.

So I suggest that we are acting on one hand as if we know much more than we really know about the operation within a particular system. I submit to you that we would do much



better if we really would pool the information and the questions that arise on the clinical level with the tremendous technical competence and brilliance that you individuals have in the laboratory. Let's apply it step by step—not to cancer in general, but to particular lesions. Because I'm a good breast pathologist, it does not follow that I'm a good bone pathologist. I suggest that this problem is much more complex than you are making it out here.

One final point in regard to these killer lymphocytes. I know of no structural representation of these in breast

cancers, which are prognostically favorable, except for the very small minority—2% of the cases with diffuse lymphoid infiltrate. On the other hand, as Dr. Herberman said, prognosis is correlated with cellular hypersensitivity which is correlated with sinus-histiocytosis reactivity of the node or perivenous lymphocyte infiltrations. No cell-cell contact is seen in such responses. In short, there's a lot that we do not know.

**Bloom:** Thank you for that healthy purgative. Now we will close this discussion with a paper by Dr. Tevethia.



# Participation of Macrophages in Tumor Immunity<sup>1, 2</sup>

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**SUMMARY**—Low numbers of lymphoid cells from BALB/c mice bearing tumor transplants of papovavirus simian virus 40 (SV40)-transformed cells can inhibit tumor development by virus-free, SV40-transformed cells in syngeneic adult recipients but not in preirradiated hosts or in animals treated with silica, a specific macrophage toxin. Normal bone marrow cells supplied the function necessary for neutralization of tumor cells by immune lymphoid cells in mice irradiated with 700 R. It is concluded that normal macrophages cooperate in some manner with immune lymphoid cells in tumor rejection.—*Natl Cancer Inst Monogr* 35: 279–282, 1972.

EVIDENCE is considerable that immune lymphocytes can inhibit syngeneic tumor cell replication *in vitro* (1, 2), but direct inhibition of tumor cells by immune lymphocytes *in vivo* has not been demonstrated except in the allogeneic system (3), in which purified thymus-derived immune lymphocytes directly prevented the replication of (DBA/2) P-815 tumor cells in heavily irradiated C3H mice.

The present work reports that cell cooperation occurs *in vivo* between immune lymphocytes and normal macrophages in the rejection of papovavirus simian virus 40 (SV40)-transformed cells in inbred BALB/c mice. This study will be detailed elsewhere (4, 5).

## MATERIALS AND METHODS

*Animals.*—BALB/c mice (4–8 weeks old) were obtained from Texas Inbred Mouse Co, Houston, Texas.

*Tumor cells.*—The mKS-A TU-5 line of SV40-transformed cells was obtained from Dr. Saul Kit. The tumor cells are free of infectious virus and virion antigen, contain SV40-specific tumor (T) antigen, and tumor-specific transplantation antigen (TSTA). Mice bearing tumors induced by TU-5 cells develop immune lymphocytes that can specifically inhibit SV40-transformed cells (4, 5).

A spontaneously transformed BALB/c line (TUT3 #2), which lacks SV40 TSTA, was also used as a control.

*Tumor cell neutralization test.*—Lymphoid cells from immune or normal donors were mixed with tumor cells in various proportions and incubated for 30 minutes at 37°C. The lymphocyte-tumor cell mixture was then inoculated into adult recipients which were watched for tumor development.

## RESULTS

The results (table 1) show that spleen cells from mice, either immunized with live SV40 virus or

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TABLE 1.—Demonstration of immune lymphoid cells in SV40-immunized and TU-5 tumor-bearing mice

Source of spleen cells	Treatment of spleen cells*	Ratio of spleen cells/tumor cells	Target cells	Tumor incidence (%)
SV40-immunized mice†	None	100:1	TU-5	0
TU-5 tumor-bearing mice‡	None	100:1	TU-5	6
TU-5 tumor-bearing mice	None	50:1	TU-5	0
TU-5 tumor-bearing mice	None	10:1	TU-5	29
TU-5 tumor-bearing mice	None	1:1	TU-5	21
TU-5 tumor-bearing mice	None	100:1	TUT3 #2	100
Normal mice	None	100:1	TU-5	91
TU-5 tumor-bearing mice	500 R	50:1	TU-5	0
TU-5 tumor-bearing mice	2000 R	50:1	TU-5	67

\* Spleen cells were exposed to 500 R or 2000 R <sup>60</sup>Co-gamma radiation immediately before incubation with tumor cells.

† Mice received 10<sup>7</sup> plaque-forming units of SV40 subcutaneously 3 times at weekly intervals.

‡ Lymphoid cells were harvested from mice which had been bearing tumors for 3–5 weeks (1.5 × 1.5 cm).

bearing tumors induced by virus-free SV40-transformed cells (TU-5 cells), prevented tumor development by TU-5 cells when inoculated together into adult BALB/c mice. At a ratio of 100 immune spleen cells per tumor cell, the tumor incidence was 6 and 0%, respectively, in recipients of spleen cells from tumor-bearing mice and SV40-immunized mice. Tumor incidence was 91% in recipients of TU-5 cells and normal spleen cells. Lymphoid cells from TU-5 tumor-bearing mice did not inhibit tumor development by TUT3 #2 cells, which lack SV40 TSTA.

The results of experiments in which the ratio of immune spleen cells to tumor cells was varied are also shown in table 1. Spleen cells from TU-5 tumor bearers prevented tumor development at ratios as low as 1 per tumor cell.

This observation, that very small numbers of sensitized lymphoid cells can inhibit tumor development by 10<sup>5</sup> TU-5 cells, led us to speculate that either the sensitized lymphocytes undergo proliferation after coming in contact with SV40 TSTA on TU-5 cells or the normal host is providing a cell which cooperates with immune lymphocytes in the rejection of syngeneic TU-5 cells. The first possibility, that sensitized lymphocytes must proliferate *in vivo*, was ruled out from the finding that the immune spleen cells irradiated with 500 R (and which could not be stimulated with phytohemagglutinin to undergo DNA synthesis) effectively inhibited tumor development by TU-5 cells (table 1). However, immune spleen cells irradiated with 2000 R lost their ability to inhibit tumor development. These results clearly show that small numbers of sensitized lymphocytes can inhibit tumor develop-

ment by TU-5 cells without undergoing further differentiation or proliferation *in vivo*.

The above observation indicated that recipients may supply a cell which cooperates with immune lymphoid cells in tumor rejection. Therefore, tumor cell neutralization tests were performed in: 1) mice preirradiated (500 R), 2) mice irradiated (700 R), and reconstituted with  $6 \times 10^6$  bone marrow cells, and 3) mice treated with 3 mg of silica administered intravenously 3 days before the tumor cell neutralization experiment.

The results (table 2) indicated that immune spleen cells did not inhibit tumor development by TU-5 cells in mice irradiated 3 days previously with 500 R, when the circulating white blood cell count was <20% of the normal level. As compared to 11% in normal mice, 80% of mice in this group developed tumors, suggesting that the recipient supplies a cell which is (or the production of which is) radiosensitive and cooperates with immune lymphocytes in tumor rejection.

To determine if the radiosensitive cell is bone marrow derived, mice irradiated with 700 R and reconstituted with  $6 \times 10^6$  bone marrow cells 3 and 14 days previously were used in the neutralization test. The results (table 2) show that immune spleen cells did not prevent tumor development by TU-5 cells in mice irradiated (700 R) and bone marrow reconstituted 3 days previously, but did inhibit tumor development in mice irradiated (700 R) and reconstituted with bone marrow cells 14 days previously. Thus, it appeared that bone-marrow-derived cells, once they are present in sufficient number in the irradiated, reconstituted mice, cooperate with immune lymphoid cells in tumor rejection.

TABLE 2.—Evidence for macrophage participation at the effector level in tumor immunity\*

Source of lymphoid cells	Target cells	Tumor incidence (%) in recipients treated with:				
		None (normal)	500 R†	700 R‡ (bone marrow treated 3 days before)	700 R‡ (bone marrow treated 14 days before)	Silica treated§
Immune	TU-5	7 (1/15)†	80 (4/5)	100 (10/10)	10 (1/10)	58 (7/12)
Normal	TU-5	100 (17/17)	100 (7/7)	Not tested	Not tested	Not tested

\* Spleen cells from TU-5 tumor-bearing or normal mice were incubated with TU-5 cells in a ratio of 50:1 for 30 minutes at 37°C and inoculated into adult recipients which received various treatments.

† Mice received 500 R <sup>60</sup>Co-gamma radiation 3 days before the experiment.

‡ Mice received 700 R <sup>60</sup>Co-gamma radiation and were reconstituted with  $6 \times 10^6$  nucleated bone marrow cells. They were used as recipients 3 or 14 days later.

§ Mice were inoculated intravenously with 3 mg of silica suspended in 0.3 ml isotonic saline 3 days previously.

|| Spleen cells from TU-5 tumor-bearing mice.

¶ Numbers in parentheses indicate the number of animals developing tumors of those animals inoculated.

To determine if it is the bone-marrow-derived macrophage which cooperates with immune lymphocytes in tumor rejection, adult recipients treated with 3 mg of silica, a specific macrophage toxin (6), were used in tumor cell neutralization tests. The combined results of experiments are shown in table 2. Of the silica-treated mice inoculated with immune spleen cells and TU-5 tumor cells in a ratio of 50:1, 58% developed tumors; whereas, of normal mice receiving the same cell mixture only 7% developed tumors.

## DISCUSSION

These results suggest that the bone-marrow-derived macrophage cooperates with immune lymphoid cells at the efferent limb of tumor immunity to SV40-transformed cells in syngeneic BALB/c mice. The evidence suggesting macrophage participation is the following: 1) Immune lymphoid cells, irradiated so they could not differentiate or proliferate, could inhibit tumor formation when inoculated with equal numbers of tumor cells into normal recipients; 2) immune lymphoid cells did not inhibit TU-5 tumor formation in newborn mice (5) known to be deficient in certain macrophage functions (6, 7); 3) tumor growth inhibition by immune lymphoid cells occurred less efficiently in mice irradiated with 500 R, a dose known to readily depress production of macrophages by bone marrow (8); 4) bone marrow cells supplied the cells necessary to cooperate with immune lymphoid cells in rejection of TU-5 tumors in irradiated (700 R) hosts; and 5) immune spleen cells were less efficient in preventing tumor growth in mice treated with silica, a specific macrophage toxin (9).

Recently, some evidence has suggested a role for macrophages in the rejection of syngeneic tumor cells. This evidence is based mostly on the *in vitro* experiments showing the participation of macrophages in tumor cell killing (10, 11) and the finding that tumor cells can induce macrophage migration-inhibition factor (MIF) production from specifically immune lymphoid cells (12, 13). Also, preparations containing MIF can prevent tumor growth when inoculated at the site of tumor cell inoculation (14). The results of the present study show that, under the experimental conditions employed, co-operation between macrophages and immune lymphoid cells could be detected *in vivo* at the effector level of tumor rejection. Macrophages are necessary for the manifestation of delayed-hypersensitivity reactions as a result of antigen-lymphocyte interaction (15). It remains to be determined whether the macrophage function at the efferent limb of tumor immunity is the same as that for delayed-hypersensitivity reactions. Furthermore, this study does not rule out that immune thymus-derived lymphocytes may also directly inhibit tumor cell replication. Possibly macrophages are recruited as a result of specific immune cell-tumor cell interaction and serve to amplify the efferent limb of rejection of weakly antigenic cells.

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## **SESSION 6**

### **Heterogenization**

**Chairmen: William E. Paul and T. Mariani**



# Immunogenicity — Heterogenization and Problems of Antigen Recognition<sup>1</sup>

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IN THIS SEGMENT of the Conference on the Immunology of Carcinogenesis, we will deal with two general problems that may have important interrelations: heterogenization and exploration of strategies to increase the immunogenicity of tumor antigens. Heterogenization is a phenomenon first described by Breyere (1) and explored by other workers (2-9). This area of investigation was initiated by the challenging observation that skin grafts obtained from animals with virus-induced tumors were rejected by syngeneic non-tumor-bearing recipients. Because of the finding that skin grafts from animals in the preleukemic phase of oncogenic virus infection were also rejected, the thesis was developed that viral-induced antigenic changes were expressed on apparently nontransformed cells.

This phenomenon offered an interesting model for the exploration of the immune response to early antigenic changes in tumor cells but had serious implications, in terms of the vast problems implicit in widespread presence of neoantigens, for the immunotherapy of established tumors.

Recent experiments suggested that heterogenization may in fact represent the consequences of an immune response of the host to a few tumor cells within the transferred skin (10). This view would essentially hold that the destruction of the graft was due to an "innocent bystander" situation resulting from the activation of immunocompetent lymphocytes by tumor antigens. As a result of work which Dr. Mariani will present today (11), she suggests that even this relatively conventional idea may not be correct and that the rejection of syngeneic skin from tumor-bearing animals is not due to the immunologic attack of thymus-dependent or radiation-sensitive lymphocytes upon tumor cells.

It is to be hoped that our discussion may allow a formulation of the key issues in evaluation of this phenomenon and in a definition of the lines of research which may clarify the nature and importance of this problem in the total economy of the host reaction to tumors.

The other main theme of this segment of the Conference will be the problem of immunogenicity and the potential means for increasing the nature and magnitude of an immune response to very weak antigens. To set the stage for this, I will briefly review some aspects of the recognition events in the activation of immunocompetent cells. In the immune response to simple antigens, immunologic recognition must be considered a complex phenomenon involving perhaps 4 or more independent steps. I speak in this instance of the antibody response to thymic-dependent antigens such as sheep erythrocytes or bovine serum albumin. These responses involve the participation of at least 3 cell types, the macrophage, or another cell with similar glass adherence properties, the thymus-derived (T) lymphocyte, and the bone marrow-derived (B) lymphocyte.

The macrophage is nonspecific in the classic immunologic sense. Nonetheless, the capacity of substances to interact with macrophage surfaces varies and the capacity of macrophages to bind antigen may have an important effect on the magnitude of the subsequent immune response. Thus, in a general sense, we may regard interaction of "processing" cells with antigen as an initial level of the control of recognition. Moreover, the locus of action of many adjuvant substances, which has not been satisfactorily established, may well involve increased activity of macrophages (12).

Turning to the cells more classically considered to be specific, the T-lymphocyte appears to occupy a central role. Thus, it is involved in the initiation and manifestation of the various forms of cellular immunity; it has a crucial and specific amplification role in the activation of precursors of antibody-

<sup>1</sup> Presented at Conference on Immunology of Carcinogenesis, held by the Oak Ridge National Laboratory at Gatlinburg, Tenn., May 8-11, 1972.

<sup>2</sup> National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.



secreting cells (B-lymphocytes) and 2 levels of recognition seem to operate in the T-lymphocyte. First, the T-lymphocyte appears to bear a cell-surface receptor with a high degree of specificity for antigen. In contrast to the situation for the receptor on B-lymphocytes, the chemical nature of the T-cell receptor is a controversial issue. Immunoglobulin, perhaps of an unusual class or integrated into the membrane in a blocked fashion, has been suggested as the T-cell-surface receptor (13-15). Other investigators, however, with techniques of apparently equal sensitivity have failed to detect surface immunoglobulin on T-lymphocytes (16-17). A striking feature of T-cell receptors is their relatively limited range of specificity. T-cells specific for haptenic groups, for example, are difficult to demonstrate whereas hapten-specific B-cells and antibody are quite common (18). Another example of their distinctive specificity range as compared to antibody is apparent from the study of histocompatibility systems of mice (19-21). T-cells appear to be specific for the product of the D region or K region subloci of the H-2 system but not for individual *H-2* specificities; antibodies, on the other hand, regularly identify individual specificities. Thus, an important element of regulation is the limited range of specificity manifested by the T-cell receptor. Indeed, many classically recognized "weak" or non-antigenic substances may be materials for which no, or only limited numbers of, T-cells with specific receptors exist. Direct evidence for this contention is still lacking because satisfactory techniques to enumerate individual T-lymphocytes on the basis of their direct antigen binding properties are not yet available.

Perhaps of equal importance in the consideration of T-cells is the level of recognition contributed by the action of histocompatibility-linked immune response (*IR*) genes. These genes, which function primarily in T-lymphocytes, have been recognized in the last several years as exerting a powerful control over the ability of animals to mount immune responses to specific groups of antigens (22, 23). The *IR* genes have been shown to be linked, in mice and guinea pigs, to the major histocompatibility locus of the species. Moreover, recent work strongly suggests that the *IR* gene specifies a cell-surface material and that blocking this surface component with specific alloantisera inhibits the capacity of the cell to be stimulated by those antigens, the response to which is controlled by that *IR* gene (24).

It has been suggested that *IR* genes code for the T-cell receptor and, although I have in this introduction considered *IR* gene products as separate entities from T-cell receptors, this possibility is by no means definitively ruled out. However, there is certain evidence which weighs heavily against the possible identity of the T-cell receptor and the *IR* gene product. For example, one of the best studied *IR* genes, the so-called *PLL* gene of guinea pigs, controls the immune response of guinea pigs to poly-L-lysine, poly-L-arginine, protamine, the copolymer of L-glutamic acid and L-lysine, and to haptenic conjugates of these substances. Although these materials share certain characteristics, they cross-react very little in terms of the elicitation of cellular immune responses (25). This strongly suggests that the *IR* gene product is not the T-cell's *prime* antigen-binding receptor. At present, we lack a clear view of the chemical nature or function of *IR* gene products but we know they are crucial to immune responses. Recently, it was established that immune responses to complex proteins may be controlled by *IR* genes (26, 27) and reports of unusual sensitivity or resistance of mice and humans of particular histocompatibility to leukemia, Hodgkin's disease and other diseases, suggest that *IR* genes may be an important factor in the control of immune responses to tumor antigens (28, 29).

The final level of recognition requiring mention, albeit briefly, is the one which has been most thoroughly studied to date—the antigen-binding receptors of B-lymphocytes. These surface antigen-binding molecules of B-cells are immunoglobulins and appear to be identical, at least in specificity terms, to the antibody which the descendent of the B-cells will secrete. Although the binding characteristics of the B-cells are reasonably clear, the mechanism by which antigen interaction with B-cell receptor leads either to activation or suppression of the cell and the precise role of the T-cell in B-cell activation are still unknown.

In terms of this antigen recognition schema, a series of questions can be framed as potential topics of fruitful research, and our panelists today will, no doubt, speak on several of these issues. Among these questions are: What is the principal limiting step in the immune response to weak antigens? Can the lack of specific *IR* genes be circumvented, particularly in the activation of effector T-lymphocytes? Is there a heterogeneity in T-lymphocytes (amplifiers and effectors) and is the specificity of effectors

different from the amplifiers? In this regard, what type of procedures will be effective in the activation of effector T-cells?

Although one cannot with certainty predict the opportunities for immunotherapy or immunoprophylaxis which our expanding understanding of lymphocyte biology and immunologic recognition may offer, it seems clear that sufficient information is now being collected to clarify in the foreseeable future the potential role of immunologic intervention in neoplastic diseases

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# Nature of Rejection of Isogeneic Skin Grafts From Mice Inoculated With a Leukemia Virus<sup>1, 2</sup>

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**SUMMARY**—Virus-associated antigens often result in partial as well as complete rejection of isogeneic skin grafts. These studies primarily concern the question of whether partial rejection results from elimination of antigenic clones or interruption of the rejection process by the development of host unresponsiveness. Approximately half of all first-set (“set” refers to number of successive grafts hosts received) grafts from leukemic BALB/c donors had evidence of incompatibility, but successive second- through fifth-set grafts were progressively accepted at increasing frequencies. When hosts were preimmunized with a single subthreshold dose of virus-induced tumor cells, initial increased reactivity also subsided in subsequent successive grafts. A similar pattern was seen when prospective skin graft recipients were repeatedly preimmunized with virus-induced tumor cells. One preimmunization elicited maximal response; multiple preimmunizations were less effective. A persisting antigenicity in partially rejected grafts taken from primary hosts was evidenced by resumption of the rejection process upon grafting onto secondary hosts. These results indicate that the skin of leukemic mice contained virus-associated antigens of a weak immunogenicity in this test system, and incomplete rejection resulted from decreased host reactivity possibly caused by virus infection. Indirect evidence suggested that leukemic cells within grafts may cause graft rejection.—*Natl Cancer Inst Monogr* 35: 289–294, 1972.

EVOCATION OF transplantation immunity is not a property restricted to the usual variety of genetically determined transplantation antigens. Acquired antigens may also serve in this capacity, as evidenced by their presence in spontaneous tumors and in tumors induced by chemicals and viruses. With oncogenic viruses, cellular antigens have usually been demonstrated only in the induced tumors, but more recent studies suggest that these antigens are also present in presumably “nor-

mal” host tissues. For various reasons, the question of ubiquity of virus-associated antigens is important in understanding how a host succumbs to a potentially immunogenic tumor. We previously reported that isogeneic skin grafts from infected animals often undergo some degree of rejection on genetically compatible hosts (1), and these observations have since been confirmed and extended by others (2–6). In general, not all skin grafts from infected donors are rejected, and of those grafts showing incompatibility with their hosts, rejection may either go to completion or cease before graft destruction is complete. In classical allograft rejection, a permanent incomplete or partial rejection is practically unknown, since all cells usually contain the same complement of genetically determined antigens.

The failure of grafts from viremic or leukemic

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donors to be completely rejected may be explained by several possibilities, any one of which need not be mutually exclusive. 1) Partial rejection could result from the destruction of isolated groups or clones of cells bearing virus-associated antigens. 2) Partial and even complete rejection may not have an immunologic basis, but could result from nonspecific factors. 3) Since such grafts contain infectious virus, infection of the host could result in additional production of virus and associated antigens leading to tolerance or paralysis of immune responses, thus halting the rejection process. 4) Skin grafts from leukemic donors may be poorly immunogenic and may fail, in various degrees, to elicit or succumb to an immune response. In this report, these possibilities were tested, and evidence indicated that skin of leukemic mice contained virus-associated antigens of a low immunogenicity, and incomplete graft rejection resulted from decreased host reactivity caused by virus infection.

## MATERIALS AND METHODS

*Skin graft donors and recipients.*—Newborn BALB/cBre mice were inoculated subcutaneously with a leukemia virus originally isolated from C3H plasma cell tumor 70429 (7). The injected material was plasma prepared by either low- or high-speed centrifugation. After weaning, the mice were examined for evidence of leukemia, *i.e.*, enlarged spleen, inguinal lymph nodes, and thymus glands. Mice with one or more of these symptoms were used as skin graft donors. Most experiments involved isogenic recipients of the BALB/cBre strain. In one experiment in which we wished to determine the origin of induced tumors, (BALB/cBre  $\times$  DBA/2<sub>e</sub>De)F<sub>1</sub> recipients were used.

*Immunization.*—In some experiments, before skin grafting, mice were immunized with virus-induced tumor cells. Depending on the design of the experiment, mice were immunized with one or more subcutaneous injections of approximately  $7 \times 10^3$  cells from a tumor induced by this virus in a BALB/cBre mouse. Mice developing progressively growing tumors from this dosage of cells were discarded. The interval between successive immunizations and grafting averaged 14 days.

*Skin grafts.*—Grafts were made by a method described elsewhere in all combinations, except male to female, and the direction of hair growth was altered with respect to that of the host (8). Bandages

were removed on the 9th postoperative day. Grafts were observed 3 times a week for the 1st month and then once a week thereafter for a minimum of 100 days. Grafts undergoing rejection were observed daily. Survival was based almost entirely on outward appearance as previously described, and all grafts were placed in 1 of 3 categories as follows: 1) complete survival, 2) partial rejection—destruction of more than 50% of the graft area, and 3) complete rejection—apparent destruction of all viable graft elements. In experiments involving successive grafts, subsequent transplants were made after all evidence of rejection had disappeared in previous grafts. Complete or partial rejection usually occurred within 20 days, and the interval between successive grafts usually fell within 3–4 weeks. The term “set” hereafter refers to the order or succession of grafts; *e.g.*, hosts bearing third-set grafts previously received 2 skin grafts on 2 separate occasions.

## RESULTS

Skin grafts from leukemic donors were made onto a group of 17 normal, nonimmunized BALB/cBre hosts. A total of 8 first-set grafts were either completely or partially rejected. Second-set grafts, showing no evidence of an expected increased immune response or second-set reaction, showed instead an increased acceptance by their hosts. Similarly, almost all third- through fifth-set grafts were accepted permanently by their hosts and only 2 were rejected (table 1). During this and similar experiments, the number of mice decreased progressively, due to the necessity of discarding mice that had developed tumors possibly induced either by virus or leukemic cells contained within the transplant. A similar experiment was then conducted, but genetically compatible (BALB/cBre  $\times$  DBA/2<sub>e</sub>De)F<sub>1</sub> hosts were used. The results were similar to those of the previous experiment: A moderate number of first-set grafts underwent some degree of rejection, whereas second- and third-set grafts gave no indication of immunization elicited by previous grafts (table 2).

Former studies showed that localized tumors arising beneath skin grafts resulted from cells of donor origin. Evidence that these grafts also contained infectious virus was demonstrated by the fact that tumors arising after prolonged latent periods were of host origin. Three systemically

TABLE 1.—Rejection of successive isogeneic skin grafts from leukemic donors on nonimmunized BALB/cBre hosts

Graft set	Total No. of grafts	Rejections	
		Partial (%)	Complete (%)
First	17	4 (24)	4 (24)
Second	13	0	0
Third	6	0	1 (17)
Fourth and fifth	5	0	1 (20)

TABLE 2.—Rejection of skin grafts from leukemic BALB/cBre mice on nonimmune (BALB/cBre  $\times$  DBA/2<sub>e</sub>De)F<sub>1</sub> mice

Graft set	Total No. of grafts	Rejections		Number of tumors adjacent to skin graft (%)
		Partial (%)	Complete (%)	
First	65	12 (18)	1 (2)	17 (26)
Second	49	8 (16)	0	13 (27)
Third	27	1 (4)	0	3 (11)

TABLE 3.—Tests on transplantation specificity of 3 tumors taken from (BALB/cBre  $\times$  DBA/2<sub>e</sub>De)F<sub>1</sub> skin graft recipients after long latent periods\*

Tumor designation	Tumor recipients		
	Nonimmune		
	DBA/2 <sub>e</sub> De-immune	(BALB/cBre $\times$ DBA/2 <sub>e</sub> De)F <sub>1</sub>	
	BALB/cBre (takes/total)	BALB/cBre (takes/total)	(takes/total)
A	0/3	2/2	4/4
B	1/4	2/4	4/4
C	0/4	3/4	2/2

\* Tumor donors were nonreactive against second- and third-set skin grafts from leukemic donors.

TABLE 4.—Rejection of successive isogeneic skin grafts from leukemic donors on BALB/cBre hosts preimmunized with a single injection of virus-induced tumor cells

Graft set	Total No. of grafts	Rejections	
		Partial (%)	Complete (%)
First	33	6 (18)	17 (52)
Second	27	10 (37)	1 (4)
Third	13	2 (15)	2 (15)
Fourth and fifth	8	0	0

growing tumors were randomly selected which had appeared after an average latent period of 120 days in third-set graft recipients. In all 3 instances, no evidence of rejection was noted in either second- or third-set grafts. All 3 tumor donors showed gross evidence of leukemia. A portion of the thymus, spleen, and lymph nodes from each donor was pooled, minced with a tissue press, and transplanted subcutaneously into: 1) BALB/cBre mice preimmunized with a DBA/2<sub>e</sub>De tumor allograft, 2) nonimmunized BALB/cBre mice, and 3) (BALB/cBre  $\times$  DBA/2<sub>e</sub>De)F<sub>1</sub> mice. The results (table 3) showed that, whereas all grew progressively in hybrids, less tumors grew in BALB/cBre mice, particularly in those preimmunized with DBA/2<sub>e</sub>De tissue. The superior tumor growth in hybrid hosts indicates that these tumors were of host origin and virus induced.

TABLE 5.—Rejection of isogeneic skin grafts in relation to number of prior immunizations with an isogeneic virus-induced tumor

Number of immunizations*	Total No. of grafts	Rejections	
		Partial (%)	Complete (%)
0	23	5 (22)	2 (9)
1	22	5 (23)	8 (36)
2-3	23	5 (22)	3 (13)
4-6	10	3 (30)	0

\* With subthreshold doses of virus-induced tumor cells.

Reduced host reactivity to subsequent successive grafts was further studied in skin graft recipients preimmunized with a single subcutaneous injection of cells from a tumor induced by the virus. About half of all first-set grafts were rejected after a single immunization; however, the number of complete rejections dwindled to zero in fourth- and fifth-set skin grafts (table 4). In another experiment, mice were preimmunized with from 1-6 successive subthreshold injections of virus-induced tumor cells. One to two weeks after the last or only immunization, these plus nonimmunized controls received a single skin graft from a leukemic donor. Again, the highest number of complete rejections was observed in mice that received a single preimmunization (table 5). Additional preimmunizations resulted in fewer complete rejections. Thus prior transplants of either skin or tumor at best produced only limited increases in host reactivity. Multiple



TABLE 6.—Rejection of isogeneic skin grafts from leukemic BALB/cBre donors on preimmunized secondary BALB/cBre hosts after partial rejection on primary hosts

Immune status of primary host*	Number of grafts	Rejections	
		Partial (%)	Complete (%)
Immunized	11	3 (27)	6 (55)
Nonimmunized	7	1 (14)	6 (86)
Control†	18	1 (6)	3 (17)

\* Immunized by prior injection of virus-induced tumor cells.

† Grafts from contralateral chest wall of primary host.

exposures appeared “tolerogenic” rather than “immunogenic,” which suggested that partial rejection of skin grafts from leukemic donors could result from loss of host reactivity. To more critically examine this possibility, skin grafts from leukemic mice that had undergone partial rejection on primary hosts were removed and transplanted onto secondary hosts preimmunized with a single sub-threshold dose of tumor cells. Controls for this procedure were skin grafts taken from the contralateral chest wall of primary hosts. The results (table 6) showed a retention of antigenicity, in that most grafts which had undergone only partial rejection on primary hosts were completely rejected by their secondary hosts. Interestingly, some control grafts of putatively normal skin from primary hosts showed evidence of rejection, thus indicating an acquired antigenicity of either viral or cellular origin.

One question of primary importance concerning the rejection of grafts from infected hosts is 1) whether the virus elicits antigenic changes in native graft cells, *e.g.*, those of the epidermis, or 2) if rejection results from destruction of antigenically altered lymphoid cells within the graft. Indirect evidence of the latter possibility was suggested by the following. During other experiments, grafts of normal BALB/cBre skin had been transplanted to histocompatible, noninfected (BALB/cBre  $\times$  DBA/2<sub>e</sub>De)F<sub>1</sub> mice. After being in place for 91–120 days, these grafts were transplanted back to 15 normal and 12 DBA/2<sub>e</sub>De-immune BALB/c hosts. Both groups rejected their grafts, but a significantly higher percentage of DBA/2<sub>e</sub>De-immune BALB/cBre hosts rejected their grafts (5/15 vs. 9/12,  $P < 0.05$ ).

## DISCUSSION

These results demonstrate the development of an immunologic unresponsiveness toward virus-associated antigens originating in isogeneic skin grafts. The repeated transplantation of either tumors or skin grafts decreased, rather than increased, host reactivity. This decrease could result from the development of enhancement- or tolerance-type mechanisms. These observations support those of Liozner *et al.* who found that pretreating hosts with virus-containing materials did not accelerate rejection of secondary “heterogenized” grafts (9). In the present study, lost reactivity may have resulted from virus infection, as unresponsiveness was demonstrated following inoculation with a similar virus (10).

The effectiveness of preimmunization depends, among other factors, on the immunogenicity of the virus and its associated antigens. Antigens associated with the virus used in the present studies were only weakly immunogenic, as demonstrated by the transplantation of its induced tumors (7) and the relatively low percentage of completely rejected skin grafts (7). Meeker *et al.* also noted considerable differences in the effects of preimmunization on skin graft rejection with different viruses (4). This factor of immunogenicity alone could account for the differences in results, ranging from no rejection to complete rejection of all grafts, reported by various investigators.

In the present study, the presence of infectious virus within skin grafts was evidenced by the development of tumors of host origin in graft recipients after long latent periods. The presence of viruses in grafts was reported by Svet-Moldavsky *et al.* (11) and Mariani *et al.* (12). The release of infectious virus and the multiplication of infected cells are two complicating variables that may cause host unresponsiveness. Therefore, to demonstrate graft rejection with virus-associated antigens of a low immunogenicity, the time relations concerning immunization and challenge are critical.

The partial rejection of grafts appeared to be a manifestation of unresponsiveness which interrupted the rejection process. This loss of host response was particularly demonstrated by resumption of rejection in formerly partially rejected grafts upon transplantation to secondary hosts. This reawakening of rejection also argues against the possibility

that partial rejection results from destruction of antigenic clones of cells within skin grafts. If the latter were true, elimination of antigenic cells would preclude rejection on secondary hosts.

Apparently not all tumors contain or elicit antigenic changes in their hosts (13), and not all oncogenic viruses share this property. An excellent example of a virus whose antigens remain somewhat localized is the mammary tumor virus (14, 15). Skin graft rejection has not been observed in mice harboring this virus, despite its apparent wide distribution in various tissues (16). On the other hand, rejection of isogeneic skin grafts can result from antigens associated with other than oncogenic viruses, as the excellent studies of Holtermann and Majde have shown with lymphocytic choriomeningitis virus (17).

The term "heterogenization" was coined by Svet-Moldavsky to imply the induction of transplantation antigens in cells of otherwise normal skin grafts (2). The present experiments, however, favor an alternate mechanism of skin graft rejection: rejection elicited by virus-associated antigens present in cells of the lymphoid series. The studies of Steinmuller and Hart showed that antigenic passenger cells can result in rejection of otherwise syngeneic grafts (18), and the present studies with grafts to and from genetic hybrids confirm their observations. Furthermore, Mariani and co-workers observed tumor cells within such grafts (6). The inherent ability of lymphoid cells to migrate may explain the reported successes with leukemia viruses and the negative observations obtained with syngeneic skin grafts from mice bearing the mammary tumor virus (14, 15).

Weaver *et al.* (19) first observed that destruction of target cells by immunocytes resulted in mutual death plus destruction of other cells in the vicinity. Therefore, the destruction of preleukemic or leukemic cells within grafts could well result in destruction of other graft elements such as epidermal cells. This possibility is particularly attractive in view of the studies of Andrew showing lymphocyte transformation in epithelium (20). Thus virus infection of lymphoid cells would result in antigenic changes in the epidermis *per se*. Either cell migration or heterogenization may account for the ubiquity of certain virus-associated antigens that elicit rejection. Studies in this area, however, are complicated by factors, such as virus replication, which alter or impair immune responses.

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# Immune Response of Mice to Concanavalin A-Coated EL 4 Leukemia<sup>1</sup>

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**SUMMARY**—Recent studies on the *in vivo* and *in vitro* immune response of C57BL/6 mice to EL 4 leukemia were reviewed. High levels of cytotoxic lymphoid cell (CLC) activity developed in spleens of mice given injections of concanavalin A (Con A)-coated, irradiated EL 4 cells and challenged with irradiated EL 4 cells. Little or no activity developed in the spleens of mice immunized twice with irradiated EL 4 cells. Spleen cells of mice initially injected with irradiated EL 4 cells responded to *in vitro* EL 4 challenge with the development of high levels of CLC activity. These findings suggested that the *in vivo* development of CLC was suppressed in EL 4-immunized mice. The nature of this suppression and the mode of action of Con A were not elucidated, though several potential mechanisms were identified. These studies may be relevant to current interpretations of the nature of the immune response to tumors and to tumors and to the development of immunotherapeutic approaches to tumor eradication.—*Natl Cancer Inst Monogr* 35: 295–299, 1972.

THE NATURE of the interaction between a growing tumor and the immune system is poorly understood. There are presently two general hypotheses to account for successful tumor growth. Both assume the existence of tumor-associated surface antigens (TASA) (1). The first hypothesis is that the TASA are weakly immunogenic and do not evoke a sufficiently strong antitumor response. The weak immunogenicity has been attributed to a failure of immunologic recognition of the TASA (2). The second hypothesis is that TASA do generate potentially adequate cellular immunity, but that “effector cells” are prevented from destroying the tumor by the interposition of anti-TASA-blocking antibodies (1, 3). These hypotheses suggest

different approaches to how best the immune system might be manipulated to eradicate tumor. Those researchers who maintain that TASA are not adequately recognized advocate the use either of adjuvants to increase the host's immunologic responsiveness (4–6) or of chemical or biologic agents to render the tumor cell more foreign (2, 7, 8). Included in this approach is the attachment to the cell of additional immunogenic moieties to serve as antigenic “carrier” determinants for the TASA (2, 7). If, however, tumors normally evoke both effector cells and blocking antibodies, then immunologic manipulation aimed at either suppressing the production, removing or inactivating these blocking antibodies would be indicated (9–11).

Antitumor effector cells are cells whose primary *in vivo* function is to locate and destroy target tumor cells. In this paper, we summarize experiments suggesting that, in tumor-immunized mice, the development rather than the *in vivo* function of cytotoxic lymphoid cells (CLC) is suppressed. Several distinct types of effector cells may exist (*see later*). The CLC described in this study are those detectable in a 4-hour *in vitro* tumor cytotoxicity assay (12, 13). Our results raise the possibility of new im-

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munotherapeutic approaches to tumor eradication, but they also indicate major areas of uncertainty regarding the nature of the immune response in tumor-bearing hosts.

### Experiments With Concanavalin A-Coated Tumor Cells

A series of experiments were designed to induce in C57BL/6 mice high levels of CLC activity against EL 4 leukemia cells. Assuming that the TASA of EL 4 cells was weakly immunogenic, we sought to enhance immunogenicity by attaching a foreign protein to the cell surface. The protein selected was concanavalin A (Con A), a plant agglutinin with affinity for the surface membrane of many neoplastic cells. Con A was viewed as possibly providing antigenic "carrier" determinants for the TASA. Accordingly, mice were given 2 intraperitoneal injections of irradiated EL 4 cells reacted *in vitro* with Con A, while control mice were given irradiated, otherwise untreated, EL 4 cells. The mice were tested for splenic CLC activity 10 days after the second injection. As reported elsewhere (14), a significant but small immune response was detected in the spleens of mice receiving 2 injections of Con A-coated EL 4 cells. No CLC response was detectable in spleens of control mice. The CLC did not cause lysis of an unrelated leukemia of BALB/c mice, LSTRA (lymphosarcoma transplant ascites) (table 1). In repeated experiments, similar

TABLE 1.—Mice given 2 injections of irradiated, Con A-coated EL 4 cells developing low or undetectable levels of anti-EL 4 splenic CLC activity (expt. A); mice given an injection of coated EL 4 cells and challenged with uncoated EL 4 cells developing high levels of anti-EL 4 splenic CLC activity (expt. B)\*

Experiment	Immunization		CLC response (% lysis $\pm$ SE)
	Primary	Secondary	
A	EL 4	EL 4	0.48 $\pm$ 0.29
	Con A-coated EL 4	Con A-coated EL 4	3.95 $\pm$ 0.70
B	EL 4	EL 4	0.42 $\pm$ 0.26
	Con A-coated EL 4	EL 4	26.64 $\pm$ 0.97

\* Mice were immunized by being given an initial injection of  $5 \times 10^5$ , 1500 R-irradiated, Con A-coated or uncoated EL 4 cells. After 3 weeks, they received a second injection of  $5 \times 10^7$ , irradiated, Con A-coated or uncoated cells. After 7–10 days, the splenic CLC activity per  $5 \times 10^6$  cells was assayed against  $5 \times 10^4$ ,  $^{51}\text{Cr}$ -labeled, EL 4 target cells. Each value represents the mean  $\pm$  standard error (SE) of 4–8 determinations. To coat EL 4 cells with Con A,  $10^7$  cells/ml were reacted with 25  $\mu\text{g}$ /ml Con A for 30 minutes at 37°C.

TABLE 2.—Mice given an injection of Con A-coated EL 4 cells and subsequently challenged with either Con A-coated (expt. A) or uncoated (expt. B) EL 4 cells developing CLC reactive with Con A-coated LSTRA\*

Target cell	CLC response (% lysis $\pm$ SE)	
	Experiment A	Experiment B
EL 4	-1.82 $\pm$ 1.00	30.24 $\pm$ 0.95
Con A-coated EL 4	13.82 $\pm$ 1.61	21.64 $\pm$ 1.22
LSTRA	0.90 $\pm$ 0.42	2.01 $\pm$ 1.91
Con A-coated LSTRA	9.79 $\pm$ 0.83	15.62 $\pm$ 4.74

\* Immunization and assay were performed similar to the protocol described in table 1. The usual low level of splenic CLC activity in mice immunized twice with Con A-coated EL 4 cells was not observed in this experiment.

low and occasionally no anti-EL 4 CLC activity was detected in spleens of mice doubly immunized with Con A-coated EL 4 cells. Significant activity was detectable, however, against Con A-coated EL 4 and Con A-coated LSTRA target cells (table 2). It was subsequently observed that high levels of anti-EL 4 CLC activity can be achieved by initially injecting mice with irradiated Con A-coated EL 4 cells and boosting with irradiated, but uncoated, EL 4 cells (15) (table 1). The immune response reacted with Con A-coated, but not uncoated, LSTRA cells (table 2).

In experiments to be detailed elsewhere, the following observations were made: 1) The concentration of Con A with which the EL 4 cells are reacted can vary from 5–500  $\mu\text{g}$ , and the number of cells injected can range from  $10^4$ – $10^6$  and still result in the development, after challenge with irradiated EL 4 cells, of high levels of CLC activity. 2) Con A given separately from the EL 4 cells (1–100  $\mu\text{g}$ ) or Con A bound to an unrelated tumor does not prime mice for a second injection of irradiated EL 4 cells. 3) Mice injected with uncoated EL 4 cells can respond to subsequent immunization with LSTRA.

Experiments of a more preliminary nature indicated that Con A-reacted EL 4 cells 1) are phagocytized more readily, 2) show reduced binding of a specific anti-H-2b antibody, 3) show reduced DNA synthesis and *in vitro* survival, and 4) markedly stimulate the *in vitro* proliferation of normal spleen cells. Whether any one of these activities enhances the immunogenicity of EL 4 cells remains to be determined. That Con A may act on the immune system, rather than exerting its effect by altering EL 4 cells, was suggested by an experiment

TABLE 3.—Development of CLC activity *in vitro* from normal and EL 4-preimmunized mice

Experiment	Preimmunization*	CLC activity before culture (% lysis $\pm$ SE)	EL 4 added to culture†	CLC activity after 5 days of culture (% lysis $\pm$ SE)‡
A	Nil	—	—	0§
	EL 4	—0.38 $\pm$ 0.98	+	6.14 $\pm$ 0.59
			—	3.51 $\pm$ 0.89
	Con A-coated EL 4	2.53 $\pm$ 1.04	+	10.55 $\pm$ 1.73
B			—	15.95 $\pm$ 2.82
			+	55.27 $\pm$ 4.62
	Nil	—	—	0§
	EL 4	—0.38 $\pm$ 0.43	+	10.40 $\pm$ 0.73
			—	14.74 $\pm$ 0.74
			+	20.83 $\pm$ 0.72

\* Mice were preimmunized by being given an injection of  $5 \times 10^5$ , irradiated, Con A-coated or uncoated EL 4 cells 3 weeks before the *in vitro* culture. The culture conditions are described elsewhere (15).

† Exposed to 2000 R radiation.

‡ The values shown represent the mean  $\pm$  SE of quadruplicate samples. The assay procedure was similar to that described in table 1, except that, after *in vitro* immunization,  $2 \times 10^5$  viable lymphoid cells were reacted with  $5 \times 10^4$ ,  $^{51}\text{Cr}$ -labeled EL 4 cells. Additional experiments have shown that *in-vitro*-generated CLC are specific for EL 4 cells.

§ The  $^{51}\text{Cr}$  release by these groups was used to calculate the CLC activity of the other groups in each experiment. It was therefore taken as zero. The actual % lysis achieved by these groups was not greater than that of labeled EL 4 cells exposed to medium in each experiment.

in which Con A-coated IM 9 cells (an immunoglobulin-secreting human cell line) were administered together with irradiated, uncoated EL 4 cells. This regimen resulted in successful priming for a subsequent injection of irradiated, uncoated EL 4 cells (15). The possibility that Con A passed from IM 9 to EL 4 cells in this experiment, however, was not excluded (15).

#### *In Vitro* Development of CLC

To define further the mechanism by which Con A-coated EL 4 cells could prime mice for a subsequent injection of EL 4 cells, we utilized the *in vitro* tissue culture technique as a preferred means for the EL 4 challenge. Spleens of normal mice or mice previously injected with irradiated or irradiated, Con A-coated EL 4 cells were cultured with or without additional irradiated EL 4 cells. It was observed that: 1) Both normal spleen cells and spleen cells from previously immunized mice respond to *in vitro* immunization with the development of specific anti-EL 4 CLC. The magnitude of the CLC response of cultured spleen cells from mice preimmunized with Con A-coated EL 4 cells is higher than that of cells from mice preimmunized with uncoated EL 4 cells, which in turn is higher than the response of normal spleen cells. 2) Prior to *in vitro* culture, no CLC activity is detectable in spleen cells of mice previously injected with uncoated EL 4; yet low levels of activity develop in

cultures when these cells are cultured without additional EL 4 cells (table 3) (15, 16).

#### DISCUSSION

These results demonstrate a striking difference in the performance of immunocompetent cells *in situ* and *in vitro*. Immunization of the host with EL 4 cells fails to elicit significant levels of splenic CLC activity. In dramatic contrast, immunization *in vitro* elicits activity. The magnitude of the *in-vitro*-generated CLC response of spleen cells from EL 4-preimmunized mice is consistently higher than that of normal cells. Further, CLC activity may develop in cultures of spleen cells of preimmunized mice in the absence of additional EL 4. These results indicate that *in-vivo*-injected mice are primed; *i.e.*, they respond immunologically to EL 4 cells. The *in vivo* response does not lead to the production of CLC, possibly because a block occurs in the maturation of CLC. What is noteworthy is that this blocking or suppressive mechanism is less evident in an *in vitro* system. Experiments are in progress to define the mechanism suppressing the *in vivo* development of anti-EL 4 CLC. Several of these experiments have involved the addition of serum from normal or EL 4-immunized mice to the tissue culture system. The spontaneous or EL 4-induced *in vitro* development of CLC has not been specifically inhibited by serum from EL 4-immunized mice.



The concept of suppressed development of effector cells appears to be in contrast to the conclusion derived from studies in which the microcytotoxicity or colony-inhibition (CI) assay was used (1, 3). These studies have suggested that effector cells are already present in tumor-bearing hosts, but that the tumor is protected from destruction by the interposition of serum-blocking factors. We are trying to resolve this issue. CI assays require an incubation of 30–40 hours, and one possibility is that, during this interval, CLC as detected in the 4-hour assay develop from noncytotoxic precursors. Experiments of a preliminary nature have suggested that colony-inhibitory activity is present in EL 4-immunized mice but cannot be attributed to the development of CLC (16). The cells mediating CI may, therefore, represent a distinct class of effector cells, or alternatively may not be effector cells.

Various *in vitro* assays are being used to assess cell-mediated antitumor immunity: These include 1)  $^{51}\text{Cr}$ -release assay for CLC; 2) CI and microcytotoxicity assays; 3) detection of the release of lymphokins, *e.g.*, lymphotoxin and migration-inhibitory factor; 4) lymphocyte blastogenesis; and 5) macrophage-mediated tumor cell destruction and growth inhibition. These different assays suggest that several distinct effector mechanisms operate in the tumor-immune host (17–22). The interrelationships, relative *in vivo* potencies, and susceptibility to *in vivo* inhibition of the cells active in these various assays need to be determined.

We are investigating the capacity of spleen cells rich in CLC activity to achieve tumor remission when adoptively transferred to tumor-inoculated mice. Similar studies are required to assess the *in vivo* function of “activated macrophages” (21, 22), colony-inhibiting cells, etc. Possibly some of the *in vitro* assays do not detect effector cells, but rather, detect cells which function *in vivo* to regulate the development of effector cells. The cellular events involved in the generation of effective immunity are poorly understood and may involve the participation of specific amplifying or suppressive cells (2, 23). These cells may, on contact with antigen, release pharmacologic mediators which function *in vivo* to regulate the maturation of CLC precursors but which, within the confines of a CI assay, may nonspecifically affect the proliferation or viability of tumor target cells. A suggestion that the cells detected in CI assay may not necessarily be effector cells is the finding that lymphocytes from

neonatally induced tolerant mice, which display CI *in vitro* (24), do not induce graft-versus-host (GVH) disease in hosts syngeneic with the tolerated strain (25). It has been argued that cells producing blocking antibody might be co-transferred with effector cells and that these antibodies suppress the development of GVH disease (26). A recently reported experiment, however, did not support this proposition. Thus, serum or thoracic-duct lymphocytes from tolerant rats could not suppress the ability of normal thoracic-duct cells to induce GVH in recipient rats (27).

Immunotherapy should be aimed at equipping the host with a potent type of effector cell. In experimental studies, maximal levels of CLC activity can, in general, be elicited only by immunization with optimal protocols. Immunization with too little or too much antigen, or with injections given too frequently, often evoke lower responses. The immune stimulus provided by a growing tumor need not be one which will evoke high levels of CLC activity. Rather, development of effective levels may be thwarted or forestalled, possibly by a suppressive mechanism. An important suggestion from our work is that such inhibition appears to be reversible and that, with *in vitro* culture techniques, CLC will develop. If returned to the tumor-bearing host, these cells may possibly be of immunotherapeutic value.

An alternative approach is to try to evoke the *in vivo* development of CLC, possibly by immunization with chemically altered tumor cells or administration of adjuvants, etc. It is clearly important that the regulatory mechanisms determining the production of CLC are defined. Of serious concern is the potential risk of evoking autoimmunity with these procedures. Thus, Chiba *et al.* administered to dogs human, gamma-globulin-coated, autologous lymphocytes in Freund's adjuvant. The dogs developed what appeared to be autoimmune lymphocyte destruction (28).

We are investigating whether the administration of Con A-coated normal tissues evokes autoimmunity in mice. The existence in neonatally tolerant (24), tetraparental (allophenic) (29), and allogeneically pregnant mice (30) of colony-inhibitory cells active against cells of “tolerated” donors suggests that CI against normal self-antigens may be present. The CI detected in tumor-bearing hosts may be a heightened degree of self-reactivity rather than a tumor-specific response. The use of fibro-

blasts as a control normal tissue in many CI assays does not exclude a reaction against organ-specific antigens. Furthermore, the wide cross-reactivity observed with CI assays against tumors of the same histologic type (37) is consistent with this hypothesis and again suggests the possible risk of evoking autoimmunity with currently proposed immunotherapeutic procedures.

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# Augmented Immunogenicity of Tumor Cell Homogenates Produced by Infection With Influenza Virus<sup>1</sup>

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**SUMMARY**—While seeking to isolate biologically effective tumor transplantation antigens (TTA's) from a mouse fibrosarcoma transformed by simian virus 40 and from fibrosarcomas induced by 3-methylcholanthrene, we found that the immunogenicity of tumor homogenates was markedly augmented if the tumor cells were first infected, or "heterogenized," with influenza virus. The plasma membrane fraction of the virus-infected tumor cells appeared to contain most of the virus-augmented TTA. A fast footpad assay for TTA is speeding the isolation work considerably, and determination of the mechanism of action of the influenza virus effect is in progress.—Natl Cancer Inst Monogr 35: 301–307, 1972.

I WISH to clarify the definition of the term "heterogenization" as we use it and also to describe 2 basically different approaches to immunotherapy in which heterogenization has been used.

In 1964 Svet-Moldavsky and Hamburg (1) applied the terms "natural heterogenization" to describe the propensity of many viruses, during natural infection, to induce in the cells that they are infecting a neoantigen which stimulates an immunologic attack on the infected cell by the host. These authors proposed a form of tumor therapy involving what they termed "artificial heterogenization of tumors" in which "... compounds or biological agents are administered which selectively accumulate or multiply in the tumors, thereby providing new antigenic determinants in the tumor cells. It is precisely on these determinants the immunological effect is produced" (2).

Our definition of heterogenization contains the complete sense of the definition of Svet-Moldavsky and Hamburg and, specifically, includes the case of a foreign transplantation antigen placed on a tumor cell *in vitro*: "...to heterogenize a cell is to cause a foreign antigen, especially a transplantation antigen, to occur on the surface of a cell, either *in vitro* or *in vivo*." The synonym "neoantigenization" clearly denotes what we mean by heterogenization.

Table 1 gives examples of the different types of antigens used to "heterogenize" cells. Thus one can couple a foreign protein to the cell, covalently or by adsorption, insert a histocompatibility antigen by fusion, alter surface structure with a chemical reaction, delete surface structure with a hydrolytic enzyme, or, of greatest interest to us, insert a foreign protein in association with infection by certain viruses.

In the past, there were 2 basically different approaches to the immunotherapy of tumors in which heterogenization was used. The first approach (text-fig. 1) was that of Svet-Moldavsky and Hamburg. It involved attempts to infect the growing tumor *in vivo* with a heterogenizing virus to stimulate an immune reaction in the host against virus-infected tumor cells. The disadvantage of this

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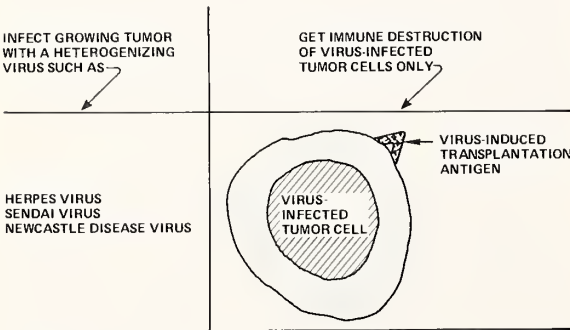
<sup>3</sup> National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

TABLE 1.—Antigens used in heterogenizing cells

Antigens	Examples	References
Foreign protein	Keyhole limpet hemocyanin (covalently bound)	(3)
	Concanavalin A (adsorbed)	(4)
Histo-compatibility antigen	HL-A antigen inserted in mouse cells by fusion	(5)
Modified self	Iodoacetate-treated cells	(6)
Deleted self	Neuraminidase-treated cells	(7)
Viral antigen	Herpesvirus, adenovirus, influenza virus, Newcastle disease virus, Sendai virus, simian virus 40, polyoma virus, oncornaviruses (Gross, Friend, Moloney, Rauscher, and mammary tumor virus)	(8-14)

method is obvious. Before becoming susceptible to immune attack, the tumor cells must become selectively infected with the virus *in vivo*.

The other approach to the use of heterogenization in tumor immunotherapy involves the hypothetical concept of the helper antigen (15). Text-figure 2 illustrates 2 examples of this approach. The first is that of Czajkowski *et al.* (3), who attempted to augment the immune response to a tumor by immunizing the host with *intact tumor cells* heterogenized *in vitro*. The second is that of Lindenmann and Klein (8), who worked with *tumor homogenates* rather than intact cells and used Ehrlich ascites tumor “heterogenized” with influenza virus. The influenza virus lysates of this allogeneic tumor immunized mice against challenge with uninfected tumor cells, whereas non-virus-infected mechanical homogenates of the tumor had no effect. Axler and

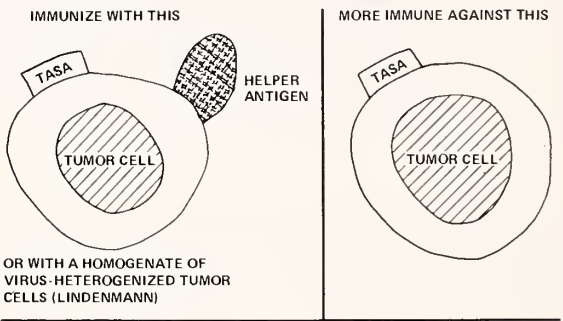


TEXT-FIGURE 1.—Heterogenization as used by Svet-Moldavsky and Hamburg.

Girardi (9) demonstrated the same type of phenomenon in a syngeneic hamster tumor system.

One readily sees that the hypothetical helper antigen is related to a tumor antigen as a carrier protein is related to a haptene in the humoral immune response. However, tumor immunity is primarily cell mediated, and haptene-carrier effects in the cellular immune system have not yet been convincingly demonstrated. Thus the actual mechanism of the augmented tumor transplantation antigen (TTA) activity in tumor homogenates after influenza virus heterogenization still needs clarification.

The major goal of our work is the isolation of purified, concentrated TTA's in a form that will immunize animals against tumor transplant chal-



TEXT-FIGURE 2.—Heterogenization involving the hypothetical concept of the helper antigen, as used by Czajkowski *et al.* and Lindenmann and Klein. TASA = tumor-associated surface antigen.

lenge. The tumor systems we are now using are a fibrosarcoma transformed by simian virus 40 (SV40) and fibrosarcomas induced by 3-methylcholanthrene (MCA) in BALB/c mice. The work was initially done with the SV40-induced tumor and is now being confirmed with the MCA-induced tumors.

The SV40-transformed tumor cells we use do not contain SV40 and, other than having produced a strong TTA with which it is convenient to work, SV40 has no further bearing on the phenomenon of TTA augmentation with the influenza virus.

The immediate obstacle one usually finds in trying to isolate TTA from tumor homogenates is the dependence of TTA activity on the viability of the tumor cells (table 2). Whereas 5 million X-irradiated tumor cells immunized perfectly against tumor challenge, a frozen-thawed homogenate of 10 times that

number of cells was not immunogenic. Table 2 also shows our experimental routine.

Table 3 shows that the TTA on the tumor cells could be stabilized to a certain extent by fixation with 1% formalin. Whereas the frozen-thawed tumor homogenate did not induce resistance to tumor challenge, tumor cells fixed with 1% formalin and then frozen and thawed induced some protection compared to that found in the controls, and the mean weight of the tumors that did occur was markedly reduced. Tumor cells frozen first and then formalin-fixed within seconds after thawing were nonimmunogenic.

The fluid mosaic model of plasma membrane structure recently proposed by Singer and Nicolson (16) is useful in the consideration of the structural basis for the viability dependence of the TTA. In this model, protein molecules move freely in the

TABLE 2.—Viability dependence of TTA of SV3T3-T4 fibrosarcoma cells\*

Inoculated with	Number of tumors
	Number challenged
X-irradiated tumor cells ( $5 \times 10^6$ )	0/20
Frozen-thawed tumor cells ( $5 \times 10^7$ )	19/20
No treatment, control	20/20

\* Inoculation: subcutaneously on days 0 and 3; challenge:  $10^6$  SV3T3-T4 cells subcutaneously on day 14; and recording: tumors scored 30 days after challenge.

plane of the membrane in a sea of phospholipid. The TTA configuration on the cell surface could represent an energy-dependent steady state that becomes disorganized when the cell dies. The steady-state pattern of the viable cell surface could be permanently fixed by 1% formalin, so that some TTA activity is retained even after homogenization.

Looking for something better than formalin fixation to preserve TTA activity, we tried homogenates of tumor cells that had been infected with influenza virus. Table 4 shows the excellent results. The important specificity control shows that animals inoculated with homogenates of normal BALB/c cells infected with influenza virus were not made resistant to tumor transplant challenge.

Table 5 shows that the influenza virus also augmented the immunogenicity of homogenates of MCA-induced tumor cells. In this experiment, the

TABLE 3.—Stabilization of TTA with 1% formalin

Inoculated with	Number of tumors	Mean tumor weight (% of control)
	Number challenged	
Frozen-thawed tumor cells ( $50 \times 10^6$ )	19/20	88
Formalin-fixed, then frozen-thawed tumor cells ( $5 \times 10^6$ )	4/10	5
Frozen-thawed, then formalin-fixed tumor cells ( $50 \times 10^6$ )	15/20	80
No treatment, control	16/20	

virus titer of the tumor cells was 100-fold lower than that in the SV40-transformed tumor cells shown above because the influenza virus stock grown in eggs had not yet adapted completely to the mouse tumor cells. We expect a stronger immunogenic effect when higher titers of influenza virus are used.

Having found a way to produce tumor homogenates with retained TTA activity approaching that of the intact tumor cells, we are now comparing various methods of purifying the TTA. One promising method is the isolation of plasma membrane fragments containing the TTA from homogenates of tumor cells infected by influenza virus. Table 6 gives some results of tests of sucrose density-gradient fractions of disrupted, virus-infected tumor cells. The 33–40% fraction, containing plasma membrane fragments and also the influenza virus, possessed substantial tumor-protective capacity. During maturation in the cell, the influenza

TABLE 4.—Augmented immunogenicity of tumor cell homogenates infected with influenza virus

Inoculated with	Number of tumors	Mean tumor weight (g)
	Number challenged	
Homogenate of SV3T3-T4 cells previously infected with influenza virus	3/35	0.125
Homogenate of SV3T3-T4 cells not infected with influenza virus	20/26	.642
Homogenate of 3T3 cells previously infected with influenza virus	15/17	.370
No treatment, control	17/19	.307



TABLE 5.—Augmented immunogenicity of MCA-induced fibrosarcoma homogenates infected with influenza virus

Group	Inoculated with	Number of tumors	
		Number challenged	Mean tumor weight (g)
I	Homogenate of MCA-induced fibrosarcoma cells infected with influenza virus	10/25	0.979
II	Homogenate of MCA-induced fibrosarcoma cells not infected with virus	21/25	1.32
III	Tissue culture medium	24/25	1.33
IV	No treatment, control	24/25	1.46

virus “heteroginizes” the entire plasma membrane with viral hemagglutinin antigen, not just the portion of the membrane that later becomes the virus envelope (17). We believe that most augmented TTA in the 33–40% fraction may exist as “heterogenized” plasma membrane microvesicles, rather than as virus with TTA incorporated into its envelope. In this experiment, the maximum possible dose of plasma membrane fragments per mouse was 40 million cells. Thus good quantitative recovery of TTA activity will be possible.

In connection with TTA isolation, we needed a faster assay for TTA activity than tumor-graft rejection, which takes 40 days. We therefore developed a useful radioisotopic footpad assay for delayed hypersensitivity to tumor cells in the mouse (10) (table 7). At the same time as the eliciting dose of tumor cells was injected into the footpad of a tumor-immune mouse, <sup>125</sup>I-labeled serum protein was inoculated intraperitoneally. The increased vascular permeability associated with the delayed-hypersensitivity reaction resulted in leakage of the labeled mouse serum protein from the vascular compartment into the inflamed area. Then 24 hours later, both feet were cut off and counted in a gamma spectrometer. The ratio of test paw counts to contralateral control paw counts is given in table 7. All necessary controls are shown. This delayed-hypersensitivity reaction against the tumor

cells was adoptively transferable with spleen cells but not with serum.

We have found that, while a homogenate of SV40-transformed tumor cells will not induce immunity to tumor transplant challenge, it will elicit a delayed-hypersensitivity reaction in animals made tumor immune either by being given a tumor that is later excised or by being inoculated with X-irradiated tumor cells (table 8). Intact, X-irradiated tumor cells immunized animals against tumor transplant challenge and also elicited a delayed-hypersensitivity reaction in tumor-immune animals. In the form of a homogenate, as has been discussed, 10 times the dose of tumor cells did not immunize against tumor transplant challenge. But a small dose of the same homogenate elicited a delayed-hypersensitivity reaction in tumor-immune animals. Finally, homogenates of tumor cells infected with influenza virus both induced tumor immunity and elicited delayed hypersensitivity. The simplest conclusion from these results is that the footpad assay is more sensitive than the tumor transplant-rejection assay and will detect amounts of TTA insufficient to immunize against tumor challenge. We are careful at this stage to distinguish 3 operationally defined immunologic determinants on the tumor cell that may or may not be structurally the same: 1) a delayed hypersensitivity-eliciting determinant, 2) a delayed hypersensitivity-

TABLE 6.—Tumor protection with plasma membrane fragments of SV3T3-T4 tumor cells infected with influenza virus

Gradient fraction inoculated	Contents	Number of tumors	
		Number challenged	Mean tumor weight (g)
<33% w/w	Lipid, few smooth membranes	8/20	0.262
33–40% (1.41–1.176)	Plasma membrane fragments	1/25	
40–45% (1.176–1.202)	Plasma membrane fragments, rough endoplasmic reticulum, ribosomes, mitochondria, lysosomes	2/24	.286
No treatment, control		16/20	.338

TABLE 7.—Delayed hypersensitivity to tumor cells in BALB/c mice demonstrated by a radioisotopic footpad assay\*

Footpad inoculum	Status of recipient	Foot-count ratio†	Average
10 <sup>6</sup> tumor cells	Immune to tumor challenge	4.08, 3.67, 3.30, 2.76, 2.58, 2.19	3.13
10 <sup>6</sup> tumor cells	Normal	1.62, 1.53, 1.27, 1.24, 1.20, 1.02	1.31
10 <sup>6</sup> syngeneic normal cells	Immune to tumor challenge	1.44, 1.34, 1.24, 1.18, 0.81	1.20
10 <sup>6</sup> tumor cells	Immunized with complete Freund's adjuvant (CFA)	1.27, 1.26, 1.24, 1.09, 1.07	1.40
10 µg <i>Mycobacterium tuberculosis</i> extract	Immunized with CFA	3.63, 3.52, 3.45, 2.24, 2.18	3.00

\* Table taken from (10).

† cpm test paw/cpm control paw: mice inoculated with <sup>125</sup>I-labeled mouse serum protein at the time of footpad inoculation.

sensitizing antigen, or the molecular configuration that primes an animal to react to the eliciting determinant, and 3) the TTA, which makes an animal immune to tumor transplant challenge.

In addition to TTA isolation, we are also investigating the intriguing question of the mechanism of the augmentation of TTA activity mediated by influenza virus. The phenomenologic situation is diagrammed in text-figure 3. VI stands for the viability-independent portion of the TTA, *i.e.*, the part that survives homogenization and will elicit a delayed-hypersensitivity reaction in tumor-immune animals. VD stands for the viability-dependent portion of the TTA, *i.e.*, the part that disappears with homogenization. The possible mechanisms for the virus augmentation of TTA are listed below.

1) The virus could act as a simple adjuvant, which would be like mixing the tumor homogenate

with CFA. When we mixed the tumor homogenate with egg-grown influenza virus, a very slight degree of tumor protection occurred, insufficient to account for the relatively marked immunogenicity of virus-infected tumor homogenates.

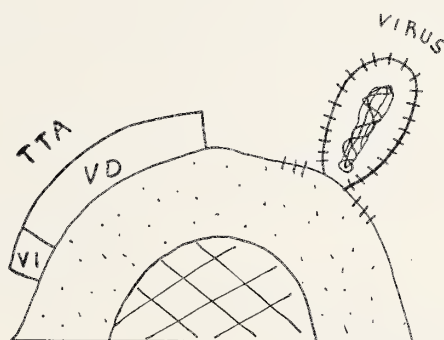
2) Infection with the virus could stabilize the viability-dependent portion of the TTA, just as 1 % formalin tends to. This is the chemical-stabilization hypothesis.

3) If the influenza virus antigens substitute for the viability-dependent portion, then a helper-antigen action could be in effect.

We have started experiments which we hope will distinguish between the chemical-stabilization and the helper-antigen hypotheses. We make

TABLE 8.—Delayed-hypersensitivity responses against intact tumor cells, homogenized tumor cells, and homogenized tumor cells infected with influenza virus

	Will immunize normal animals against tumor transplant challenge?	Will elicit delayed-hypersensitivity reaction in tumor-immune animals? (0.5 × 10 <sup>6</sup> cells)
Intact tumor cells	Yes (5 × 10 <sup>6</sup> cells)	Yes
Homogenized tumor cells	No (50 × 10 <sup>6</sup> cells)	Yes
Homogenized tumor cells infected with influenza virus	Yes (5 × 10 <sup>6</sup> cells)	Yes



TEXT-FIGURE 3.—Mechanism of the augmentation of TTA activity mediated by influenza virus.

TABLE 9.—Neuraminidase activity of influenza virus, Newcastle disease virus, and *Clostridium perfringens* preparations

Tumor cell	Total sialic acid μmoles/10 <sup>6</sup> cells	Percent of total sialic acid removed		
		Influenza virus neuraminidase, 0.02 U/ml*	Newcastle disease virus neuraminidase, 0.02 U/ml	<i>C. perfringens</i> neuraminidase, 0.9 U/ml
SV3T3-T4	6 × 10 <sup>-</sup>	27%	34%	87%
MCA-11	65 × 10 <sup>-</sup>	20%	13%	80%

\* One unit of enzyme removes 1 μmole of sialic acid from 1 mg neuraminlactose in 1 minute at 37°C when the incubation is done in 1 ml water containing 60 μmoles Tris HCl, pH 5.4, and 1 μmole CaCl<sub>2</sub>.

animals tolerant to egg-grown influenza virus with Cytoxan and then immunize them with homogenates of virus-infected tumor cells. If the virus acts as a helper antigen, it will not be recognized in virus-tolerant animals and, therefore, a virus-infected homogenate will not be immunogenic. If the virus works through a stabilizing mechanism, homogenates of virus-infected cells will still be immunogenic in the virus-tolerant animals.

4) Another possible mechanism of action related to the helper-antigen idea is that the neuraminidase of the influenza virus could modify the host cell membrane structure to produce a nonself transplantation antigen. Our current studies on a possible neuraminidase mechanism are shown in tables 9 and 10. The influenza virus strain we used possessed a neuraminidase which appeared to remove the sialic acid on the tumor cells as efficiently as the neuraminidase of Newcastle disease virus (table 9).

The *C. perfringens* enzyme (table 9) is plentiful, so we could obtain high enough concentrations to

determine the maximum amount of sialic acid that could be removed from the tumor cells. We make our own influenza virus and neuraminidase preparations and did not have enough at the time to measure the maximum hydrolyzable neuraminic acid of the cells with these enzymes. We have tested the neuraminidase hypothesis using the *C. perfringens* enzyme (table 10). A homogenate of cells infected with influenza virus showed its usual excellent protection. A homogenate of tumor cells, previously incubated for 1 hour at 37°C with a concentration of neuraminidase known to remove 87% of the total cell sialic acid, was not immunogenic.

The long-term goal of this work is, of course, to evaluate the possibility that biologically active, virus-augmented TTA could be prepared from human tumors removed at surgery and then used to immunize the patient against the development of residual metastases or recurrence of the primary tumor.

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TABLE 10.—Immunogenicity of homogenates of tumor cells treated with neuraminidase\* (*C. perfringens*)

Inoculated with	Number of tumors
	Number challenged
Homogenate of tumor cells infected with influenza virus	0/25
Homogenate of neuraminidase-treated tumor cells	23/25
Neuraminidase alone	24/25
No treatment, control	24/25

\* Neuraminidase treatment: Cells incubated 1 hour at 37°C with a concentration of *C. perfringens* neuraminidase known to remove 87% of total cell sialic acid (maximum amount possible).



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# Tumor Cell-Related Syngeneic Skin Graft Rejection in Gross Leukemia<sup>1, 2</sup>

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**SUMMARY**—Using Gross passage A (GPA) virus lymphoma and C3H/Bi and (C3H/Bi × DBA/2)F<sub>1</sub> inbred mice, we studied the phenomena of syngeneic skin rejection. Skin from GPA tumor-bearing animals is regularly rejected by syngeneic recipients and tumor regularly develops at the graft site. To uncover the causal agent and the mechanism of this rejection, we performed a series of experiments. Quantitation of tumor cells, grafting of irradiated lymphomatous and preleukemic skin, direct injection of purified GPA virus, and exchange of normal skin and skin from animals given virus injections revealed that the causal agent is the tumor cell itself, not the virus. Thymectomy and irradiation demonstrated that the immune system is not involved. We postulate that the rejection is not a classic rejection but rather results from competition between normal and tumor cells.—*Natl Cancer Inst Monogr* 35: 309–320, 1972.

IN 1963, Feldman, Gross, and Dreyfuss (1) opened a new field of investigation when they discovered virus particles in the mammary glands of pregnant, nonleukemic C3H females given injections of Gross passage A (GPA) virus. Their findings suggested that normal tissues are infected with virus to varying degrees and that antigens of “normal tissues” are altered by the virus. Since then, many investigators have explored the relationship between virus and normal tissues.

Breyere and Williams in 1964 (2) used the syngeneic skin graft model because it could be experimentally manipulated so easily. They observed

graft rejection by normal syngeneic recipients approximately 3 weeks after transplantation of skin taken from leukemic BALB/c mice given neonatal, subcutaneous injections of a virus isolated from a C3H plasma cell tumor. They also found that the graft was rejected more frequently in mice previously immunized with tumor cells than in nonimmunized recipients. To explain their findings, they postulated that the virus induced some kind of antigenic alteration of the normal tissues and this alteration caused the rejection of the syngeneic skin.

In 1967, Mkheidze and Liozner (3) and Svet-Moldavsky *et al.* (4) reported findings similar to those of Breyere and Williams and gave a name to the phenomenon of antigenic alteration of normal tissues: “heterogenization.” Mkheidze and Liozner and Svet-Moldavsky *et al.* had worked with skin isografts taken from animals bearing a wide variety of transplantable tumors, including both chemical- and virus-induced tumors. Their findings suggested that—at least in their system—the heterogenization could be attributed to a nononcogenic passenger virus somehow related to the oncogenic virus.

Many others also explored this relationship be-

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TABLE 1.—Changes in antigenicity of normal tissues: A survey of the literature\*

Investigators	Skin transplantation					
	Tumor or carcinogen†	Tumor-bearing donor	Normal recipient	Rejection (days, mean)	Postulated causal agent	Explanatory hypothesis
Breyere and Williams (2), 1964	C3H plasma cell tumor	BALB/cDe	Syngeneic	18‡	Virus	Antigenic alteration of normal tissue
Ben-Hur <i>et al.</i> (7), 1966	Ehrlich ascites tumor	Outbred mice	Allogeneic	14.2	Not specified	Antigenic loss
Eichwald and Rud (5), 1968	Spontaneous mammary carcinoma	C57BL/A	Syngeneic	None		
		BALB/c	"	"		
		DBA/2	"	"		
	BP-induced fibrosarcoma	(C57BL/10 × BALB/c)F <sub>1</sub>	"	"		
		Strain not specified	"	"		
		C57BL	"	"		
Holtermann and Majde (11), 1969	Leukemia (X irradiation)					
	LCM	SWR/J	Syngeneic	14	Virus	Antigenic alteration of normal tissue
Kaliss and Suter (6), 1968	DBA-induced sarcoma	A/J	Allogeneic	1 or 2 days' delay	Tumor in donor	1) Antibodies directed against tumor antigens in normal tissues will account for both accelerated and delayed rejections
	Leukemia (X irradiation)	DBA/2 A/J	C57BL/Ks	Accelerated (time not specified)		
	MCA-induced sarcoma	DBA/2	"	No significant changes		2) Immunologic enhancement mediated by autoantibodies may account for delayed rejections
	Spontaneous mammary carcinoma	A/H2J C3H/HeJ	"	"		
Marchant (8), 1966	DMBA-induced carcinoma	NZY	Allogeneic A	1 or 2 days' delay	Tumor in donor	Antigenic properties of skin modified by tumor (antigenic loss?)
	DBA-induced breast tumor	IF	"	"		
	Spontaneous sarcoma	IF	"	"		
	MCA-induced sarcoma	(C57BL × IF)F <sub>1</sub>	IF	4-7 days' delay		

Mariani <i>et al.</i> (34-43), 1968, 1970, 1971, 1972	GPA lymphoma	(C3H/Bi X DBA/2)F <sub>1</sub> C3H/Bi	Syngeneic	12†	Tumor cell in trans- planted skin	1) Competition between tumor and normal cell for physiologic and physical requirements (vas- cular, nutritional, spatial) 2) Products formed by tumor cells
Mathé (9, 10), 1967	DMBA	(DBA X BALB/c)F <sub>1</sub>	Syngeneic	Time not specified	Virus	Antigenic alteration of normal tissue
	Friend leukemia	(DBA/2 X CBA)F <sub>1</sub>	"	"		
	Rauscher leukemia	BALB/c	"	"		
McCarthy and Russfield (18, 19), 1967, 1968	Ehrlich ascites carcinoma	CFW Swiss (randombred)	Allogeneic CFW	Accelerated and delayed	Not specified	1) Accelerated rejection of allo- grafts results from a quantita- tive change 2) Isograft rejection indicates a qualitative change produced in host's tissues by tumor
		C3H C3H	Allogeneic A/J Syngeneic	Accelerated 8, 9; 11-15		
Meeker <i>et al.</i> (12, 13), 1967, 1968	Friend leukemia 334-C leukemia	C57BL/Ha, Ha/ICR	Syngeneic "	28 None	Virus	Antigenic alteration of normal tissue
Mkheidze and Liozner (3) and Svet-Moldavsky <i>et al.</i> (4, 14-17), 1967, 1968, 1970	DMBA-induced sarcoma  Hemocytoblastosis (X irradiation)  Moloney leukemia  SB-1 sarcoma  Hepatoma-22  Ehrlich carcinoma	C57BL/6J  C57BL/6J  BALB/c  BALB/c  C3HA  C57BL/6J	Syngeneic  "  "  "  "  "	6, 7; 9-11†  None  "  "  "	Virus	Antigenic alteration of normal tissue

\* This table is only a survey of the published data on antigenic alteration of normal tissues (skin grafts) in mice. The diversity and the differences in the table reflect the many unresolved questions surrounding heterogenization.

† BP, benzo[a]pyrene; ICM, lymphocytic choriomeningitis; DBA, dibenz[*a,h*]anthracene; MCA, 3-methylcholanthrene; and DMBA, 7,12-dimethylbenz[*a*]anthracene.

‡ Tumor development at the graft site (days after grafting): Breyere and Williams, 46 days; Mariani *et al.*, 14 days; Svet-Moldavsky *et al.*, 9 days.

tween virus and normal tissues (3-19). They worked with both isografts and allografts and used diverse animal and tumor systems (table 1). Only one group [Eichwald and Rud (5)] found no skin graft rejection. Despite the diverse systems, all the explanatory hypotheses advanced by these investigators (save Eichwald and Rud) were closely interrelated. Kaliss and Suter (6) postulated that enhancing antibody accounted for delays in allograft rejection when donors were tumor-bearing animals. Ben-Hur *et al.* (7) and Marchant (8) postulated an antigenic loss to account for similar observations. Mathé (9, 10), Holtermann and Majde (11), and Meeker *et al.* (12, 13) all observed rejection of syngeneic skin taken from tumor-bearing donors and all postulated, along with Breyere and Williams (2), Mkheidze and Liozner (3), and Svet-Moldavsky *et al.* (4, 14-17), virus-associated antigenic alteration of normal tissues as the basis for the skin rejection. In effect, all these investigators theorized that some altered antigenicity of normal tissues occurred in tumor-bearing animals. Such phenomena could be collectively considered to be forms of heterogenization.

All these investigators explored the possibility of antigenic alteration *in vivo*. Other investigators (20-22), including Hellmann and Duke (23) and Guttman *et al.* (24), carried out *in vitro* analyses and obtained evidence compatible with the heterogenization concept, that an antigenic alteration or antigenic conversion of normal tissues may be induced under appropriate conditions.

A number of investigators also studied the impact of malignancy on graft survival patterns in human cancer patients (25-33). They observed the influence of a malignancy in either recipients or donors on skin graft survival patterns. The observations in the human studies are consistent with those in the mouse studies in which the concept of heterogenization was put forward. The major graft survival pattern change seen in the human studies was a prolongation of the survival of skin allografts taken from cancer patients. Three hypotheses were advanced to explain this increase in graft survival time: antigenic loss, enhancing antibody, or impaired immunologic mechanisms.

We extensively investigated heterogenization in our own system (3, 34-43). Our work has been unique in its development of an experimental model which has clinical application. The tumor growth at the graft site and graft rejection patterns

in our system closely parallel some of the known characteristics of *lymphoma cutis* in human patients (44). Potentially our model may aid in the attempt to understand and delineate this disease.

Though the heterogenization phenomenon has been explored greatly, only two extensive investigations of this phenomenon within the confines of a specific system have been carried out—the studies of Svet-Moldavsky *et al.* and our own studies. The studies of Svet-Moldavsky *et al.* so far have largely supported the heterogenization hypothesis. In diametric opposition, our own work has yielded results that completely disprove the validity of the heterogenization hypothesis and completely eliminate consideration of the virus as a causal agent for syngeneic skin graft rejection in the GPA virus system.

## MATERIALS AND METHODS

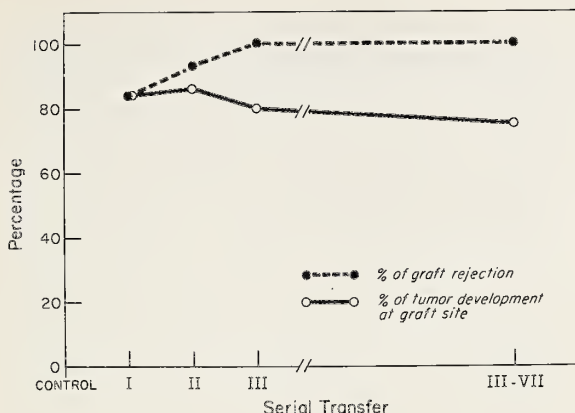
An experimental mouse system was used which included the inbred C3H/Bi, DBA/2, and F<sub>1</sub> hybrids, originally obtained from the colonies of the late Dr. John J. Bittner and Dr. Carlos Martinez; rigorous inbreeding procedures were always followed. Both strains of mice were foster-nursed on C57BL to eliminate the mammary carcinoma milk-borne agent. The GPA virus was obtained from Dr. Ludwig Gross in 1962.

We developed a method for growing the Gross lymphoma in the ascites form in both the C3H/Bi and (C3H/Bi × DBA/2)F<sub>1</sub> strains. This method has already been detailed in (45, 46). Transplantation of abdominal skin grafts was always male-to-male and female-to-female. The specific procedure is described in (35). Criterion for acceptance was luxuriant hair growth. The criteria for rejection were little or no hair growth or complete sloughing of the graft. In all experiments, a piece of the donor's skin was fixed in 10% formalin and processed by routine histologic methods for light microscopy examination. When the animals died spontaneously or when they were killed, the spleen, thymus, and inguinal, axillary, and mesenteric nodes were removed and processed for routine histologic examination.

## RESULTS

The initial problem when we began this study 4 years ago was to determine whether syngeneic skin





TEXT-FIGURE 1.—Serial syngeneic skin transplants from (C3H/Bi X DBA/2)F<sub>1</sub> donors with Gross lymphoma.

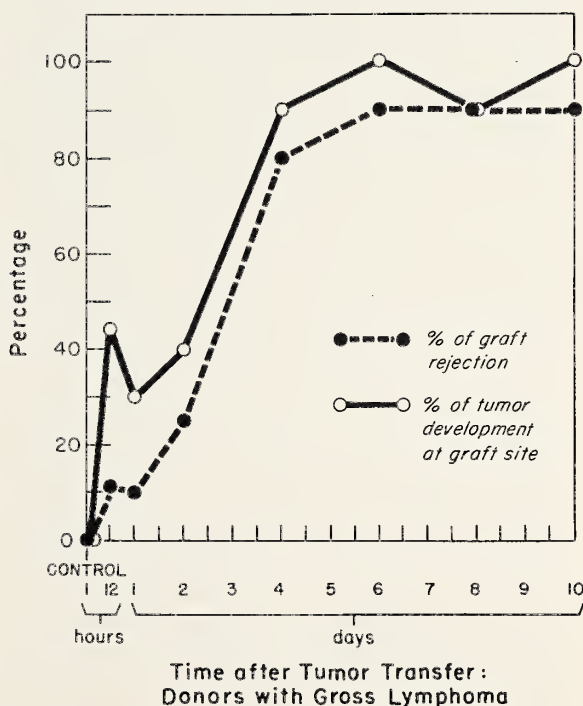
rejection would occur in our system. We took skin from animals that had fully developed lymphoma (6th through 13th ascites passage) and transplanted it onto normal syngeneic recipients (35). The skin was regularly rejected (91%; 43/47 animals) 12 days after grafting, and tumor regularly developed at the graft site (83%; 39/47 animals) 14 days after grafting. Approximately 3 weeks after grafting, the animals died of a disseminated lymphoma. Basically the same results were obtained throughout 7 successive transfers. Text-figure 1 summarizes these results. A typical rejection and tumor development at the graft site are shown in figure 1.

At this point in the study, we had 4 basic hypotheses to explain these results (35): 1) The virus altered the antigenicity of the normal tissues—the heterogenization hypothesis—and an immune reaction against altered syngeneic tissue followed; 2) the virus itself in the graft was the foreign antigen and induced an immune system-based reaction, *i.e.*, rejection of the graft; 3) the tumor cell in the transplanted skin was the basic foreign antigen that provoked the graft rejection; and 4) the tumor cell compromised the viability of the normal cell components of the syngeneic skin graft. However, the primary or working hypothesis was heterogenization. So we proceeded to an experiment which we hoped would tell us when this heterogenization occurred (text-fig. 2). We injected GPA virus in the ascites form into normal recipients. Then at serial time intervals, from 12 hours to 10 days after this injection (20th through 30th ascites passage), we took skin from the tumor-bearing animals and grafted it onto other normal recipients

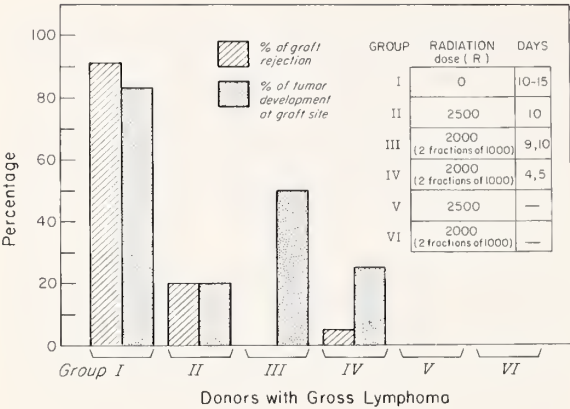
(94 animals). Our results indicated that the heterogenization must have occurred very soon after inoculation (36, 38). Some of the skin taken only 12 hours after injection was rejected (17%). But at least 4 days' exposure to the virus was needed before the skin would be regularly rejected (80%) and tumor would regularly develop at the graft site (90%).

As mentioned earlier, we routinely fixed samples of donor skin for histologic processing. Examination of the skin used in the first series of experiments—that taken from animals with a fully developed lymphoma—revealed many tumor cells. (Figure 2 is an example of one of these skin samples.) The donor skin samples from this kinetic experiment were also examined, and once again tumor cells were found. But our experimental problem was analyzing the impact of virus on the skin's antigenicity. So we devised experiments which would eliminate the tumor cells from the skin. Thus we could study the effect of the virus without interference from another agent.

The first procedure used to eliminate the tumor cell from the skin was total-body X irradiation (38). Initially, a massive dose (2500 R) was administered



TEXT-FIGURE 2.—Kinetics of syngeneic skin graft rejection and tumor development.



TEXT-FIGURE 3.—Effect of total-body X irradiation on graft rejection and development of tumor.

on the day of transplantation to the donor, which had been inoculated 10 days previously with an ascites form of GPA virus and tumor cells. That particular dose of irradiation was chosen as a starting point because it was large enough to eliminate the tumor cells but not affect the virus. Over 4,500,000 R is needed to destroy GPA virus (47). Unfortunately, even 2500 R was so strong that it greatly damaged skin tissue, and graft acceptance or rejection was difficult to interpret. So, for the second phase of the irradiation model, the dose was administered in 2 fractions. One group of animals was given 1000 R each on days 4 and 5 after tumor transfer. The second group was irradiated with the same dose on days 9 and 10 after tumor transfer. Figure 3 illustrates tumor cell degeneration in irradiated skin. This procedure left the normal cells substantially unaltered. In all, 60 animals were grafted during the various manipulations. None of the skin irradiated on days 9 and 10 was rejected, but 50% of the grafted animals developed graft-site tumors. Only one of the skin grafts taken from the animals irradiated on days 4 and 5 was rejected, and only 25% of the grafted animals developed tumors. Text-figure 3 summarizes the results. These experiments clearly showed that, if tumor growth is delayed as it was by these irradiation procedures, the graft from tumor-bearing animals will be accepted; and this delay in tumor growth is consistent with published data for irradiated mouse lymphoma cells (48).

As a corollary to these experiments, we irradiated ascitic fluid *in vitro* at 5000 R and then injected this fluid into a different group of normal animals.

Ten days later, we grafted skin from these treated animals onto yet another group of 26 normal animals. These animals were observed for over a year. Not only were there no rejections and no graft-site tumor development, but the grafts never changed during the entire observation period (38).

Our second procedure involved transplantation of “preleukemic skin” which we assumed would be virtually free of tumor cells (38). This skin was taken from 4- to 5-week-old animals that had been given injections as neonates of GPA virus (cell-free filtrates from leukemic tissues) but had no macroscopic or microscopic signs of lymphoma. In all, we grafted 49 animals with this preleukemic skin. Of these mice, 45 accepted the graft, and only 4 rejected it. None of the animals developed graft-site tumors, but all ultimately died of leukemia. We had in this experiment successfully induced leukemia in weanling mice (43). Table 2 summarizes the data. These data clearly show that GPA virus-induced lymphoma can be transferred by horizontal passage of the virus to immunologically mature animals if the virus is presented in an appropriate manner.

These 2 experiments marked a turning point in our basic conception of syngeneic skin rejection. Up to this point, we had assumed that a virus-induced heterogenization of the skin caused the re-

TABLE 2.—Incidence of Gross leukemia and bilateral thymus enlargement in weanling C3H/Bi inbred mice by preleukemic skin transplantation

Group	Development of leukemia		Thymus enlargement	
	Range in days	Mean in days	Percent	Total No. (%)
I*	95-319	174	100	41/49 (84)
II†	66-179	87	100	19/20 (95)
III‡			0	0/10 (0)
IV§			0	0/15 (0)

\* C3H/Bi mice (4 weeks old) transplanted with skin taken from 4- to 5-week-old preleukemic syngeneic mice.

† C3H/Bi mice inoculated intrathymically with 0.05 ml of 20% cell-free filtrates from leukemic tissues when 2-5 days old.

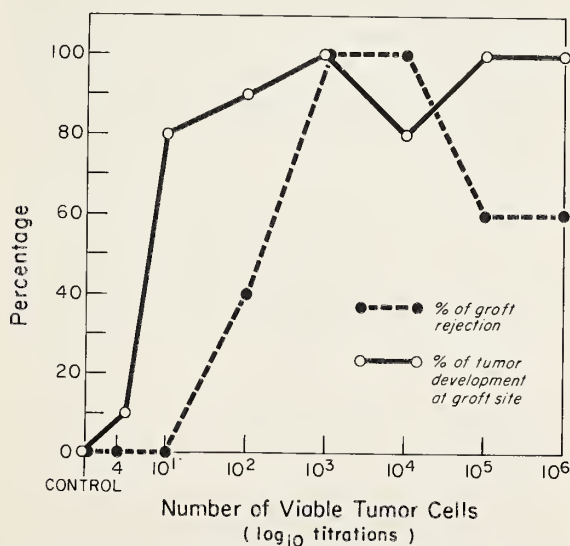
‡ C3H/Bi mice (4 weeks old) transplanted with skin taken from 4-week-old normal syngeneic mice.

§ C3H/Bi mice inoculated intraperitoneally with 0.1 ml of 20% cell-free filtrates from leukemic tissues when 4-8 weeks old.

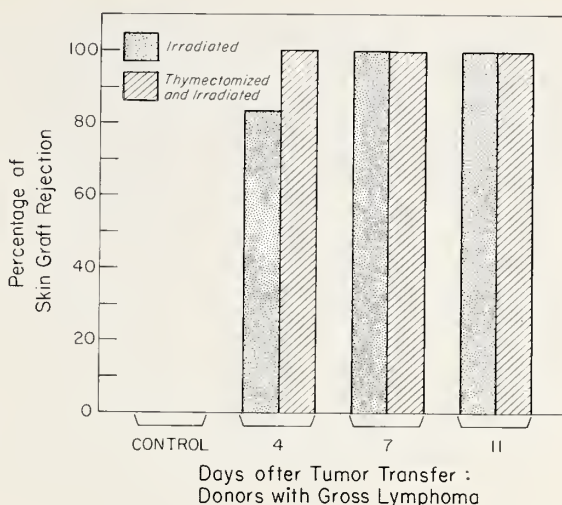
jections. For both of these experiments involving elimination of the tumor cell, precautions were taken to preserve the integrity of the virus. We had to assume that the virus was present and capable of acting throughout both of these procedures. But contrary to our expectations, we did not obtain rejections. Rather we obtained evidence suggesting that the tumor cell was more important to the rejection phenomenon than was the virus. Granted, 4 of the animals grafted with preleukemic skin rejected that skin. But preleukemic skin is only presumed to be free of tumor cells. These 4 rejections indicate that even in preleukemic skin there may be a few tumor cells. But the absence of graft-site tumors indicates that the number of tumor cells present must be very small.

These experiments cast doubt—at least for us—on the heterogenization hypothesis. They caused our focus to shift to our 2 original hypotheses dealing with the tumor cell. We proceeded from these experiments to the analysis of the role of the tumor cell in this phenomenon.

This new phase of the investigation began with the search for a correlation between the number of tumor cells in the skin and the frequency of rejection (39, 40). We prepared suspensions containing known quantities of viable tumor cells and injected them directly into newly placed grafts (50 animals). With the suspensions containing 4 and 10 cells each, we obtained no rejections (text-fig. 4). With 100



TEXT-FIGURE 4.—Quantitation of tumor cells: Effects on syngeneic graft rejection and tumor development.



TEXT-FIGURE 5.—Effect of immunosuppression on syngeneic skin rejection.

cells 40% rejections occurred, and with 1,000 cells 100% rejections occurred. The regular rejection rate continued with the higher concentrations of tumor cells. We saw tumor development with all the suspensions. At a 4-cell concentration, tumor frequency was only 10%. (This parallels the findings with preleukemic skin—8% rejection.) But from the 10-cell level up, the development was regular—ranging from 80–100%. In other words, at least 100 cells are needed before rejection occurs at all and at least 1,000 cells must be present for the rejection to become regular.

For these quantitative experiments, we used titrated ascitic fluid. We assumed that virus had been present in all the suspensions. Apparently the tumor cell itself—in sufficient concentration—and not the virus was the key factor determining whether syngeneic skin would be rejected. We further theorized that the immune surveillance system of the recipient animals was reacting to the tumor-cell-infiltrated skin as if it were foreign. Perhaps the rejection of the infiltrated syngeneic skin was basically similar to the rejection by the immune system of any allogeneic skin.

Our second approach to the function of the tumor cell in this phenomenon then was to explore specifically the relationship between the immune system and syngeneic skin rejection (41, 42). We used 2 procedures to inhibit the immune systems of the recipient animals: neonatal thymectomy (49)



and total-body X irradiation (400 R) on the day of transplantation (text-fig. 5). The recipients were divided into 3 groups: Group 1 was thymectomized; group 2 was given total-body X irradiation; and group 3 was both thymectomized and X-irradiated. After treatment, 105 of these treated animals were grafted with lymphomatous (51st through 52d ascites passage) syngeneic skin, and 29 were grafted with allogeneic (A/Bi strain) skin. Our results completely disproved our own allograft theory: The immunosuppressed animals grafted with the lymphomatous syngeneic skin regularly rejected the skin (83–100%) and developed tumor at the graft site (100%), but all the treated animals grafted with allogeneic skin accepted it.

This experiment eliminated not only the allograft hypothesis but also the consideration of the immune system and altered antigenicity as agents in the rejection phenomenon. However, it left the role of virus as unclear as it had been initially. We decided to see if purified virus had any involvement in the phenomenon.

We used 2 preparations of purified virus: cell-free filtrate from leukemic tissues and irradiated (5000 R) GPA ascites lymphoma. We followed 2 procedures for introducing the virus suspensions into the animals. In the first procedure, we injected subdermally 0.1 ml of GPA virus directly into newly placed grafts. The 50 animals were closely observed for over 1 year. During that time, no rejection occurred, and no tumor developed at the graft site. Figure 4 shows one of these animals 1 year after grafting and injection of the virus. Four of the animals died. Autopsy confirmed that they died of a disseminated lymphoma. In the second procedure, a massive dose of the virus was administered in 3 separate injections over a period of 2 weeks. Twenty-four hours after transplantation, 0.1 ml was injected directly into the skin graft. One week later, 0.1 ml was injected away from the graft into the lower right quadrant of the back. A similar dose was administered on the opposite side 2 weeks after transplantation. We still are observing these animals. So far we have seen neither rejection nor tumor development at the graft site.

If heterogenization of skin occurred, it would be assumed that the skin of the experimental animals given injections of virus over a year ago would have undergone heterogenization. It would also be assumed that this change would make impossible an exchange of skin between these animals and nor-

mal animals. Both groups of animals would reject each other's skin. We have performed some of these exchanges. So far, all the animals in both groups accepted the grafts and showed no evidence of tumor development at the graft site.

## DISCUSSION

The leukemogenic properties of GPA virus are not in question. But this virus plays no role whatever in syngeneic skin rejection in our system. Our work with the quantitation of the tumor cell, with the grafting of irradiated-lymphomatous and preleukemic skin, with the direct injection of purified virus, and finally with the exchange of normal and what ought to be "heterogenized" skin has definitely eliminated the virus as a causal agent of syngeneic skin rejection.

The heterogenization hypothesis rests on the assumption of an immune system involvement. Our work with immunosuppressive techniques completely undermined this assumption. From all our evidence, we are left with no alternative but to reject the heterogenization hypothesis.

Rather what we have discovered is that the tumor cell itself is in some way the prime agent in the rejection mechanism—our fourth original hypothesis. The precise nature of the tumor cell influence is, however, unclear. We can suggest some hypotheses. Perhaps the concept of cell competition is the key to understanding the tumor cell influence; the normal cells in the skin graft may be unable to compete effectively with the tumor cell for survival. The tumor cell may encroach upon the integrity of the normal cell. We have extensive evidence of the growth pattern of the tumor—from nodule, to a coalesced state, to a dense plaque-like infiltrate—that would support this hypothesis. The metabolic processes of the normal cell may be compromised. Or the tumor cell may deprive the normal cells of needed nutrition and vascularization and in essence "starve the cells to death." Another hypothesis—one that is the most speculative and will remain so until more is known about the tumor cell itself—is that the tumor cell may produce substances (inhibitory secretion, enzyme, cytotoxic factor) that, within the environment of the skin graft, may injure or even destroy the normal cells. Only further investigations carefully focused on the tumor cell will indicate which of these propositions—if

any—describes correctly the influence of the tumor cell.

This study of heterogenization was a fascinating and challenging investigation. It has also been highly productive. We have branched out from this study into explorations of the induction of malignancy in weanling mice—something that had rarely occurred until the unique method of introducing malignancy by means of skin grafts was tried as part of one of these experiments. We have also obtained, by carefully analyzing the tissue slides we have taken from all the animals involved in this study, some very unusual and highly provocative information concerning germinal center formation. In the face of the new discoveries that have come from this investigation, it is only a gentle irony that the final outcome of all this research was the disproval and rejection of the initial hypothesis of heterogenization and the return to the basic question that opened the whole project: “What is syngeneic skin graft rejection?”

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FIGURE 1.—Syngeneic skin graft rejection and tumor development at graft site.



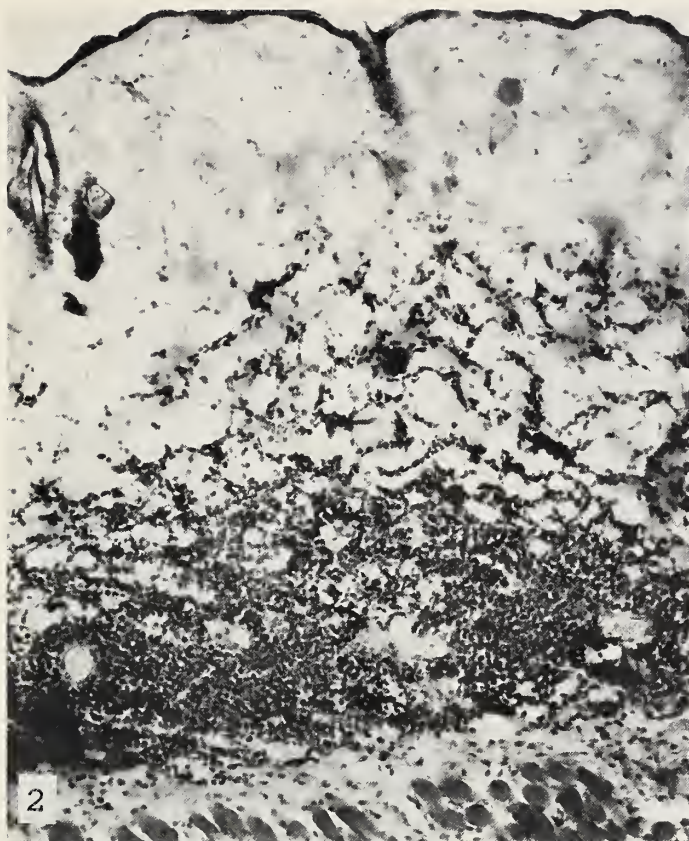


FIGURE 2.—Photomicrograph of skin taken from mouse with Gross lymphoma showing tumor cells within dermis.  $\times 100$

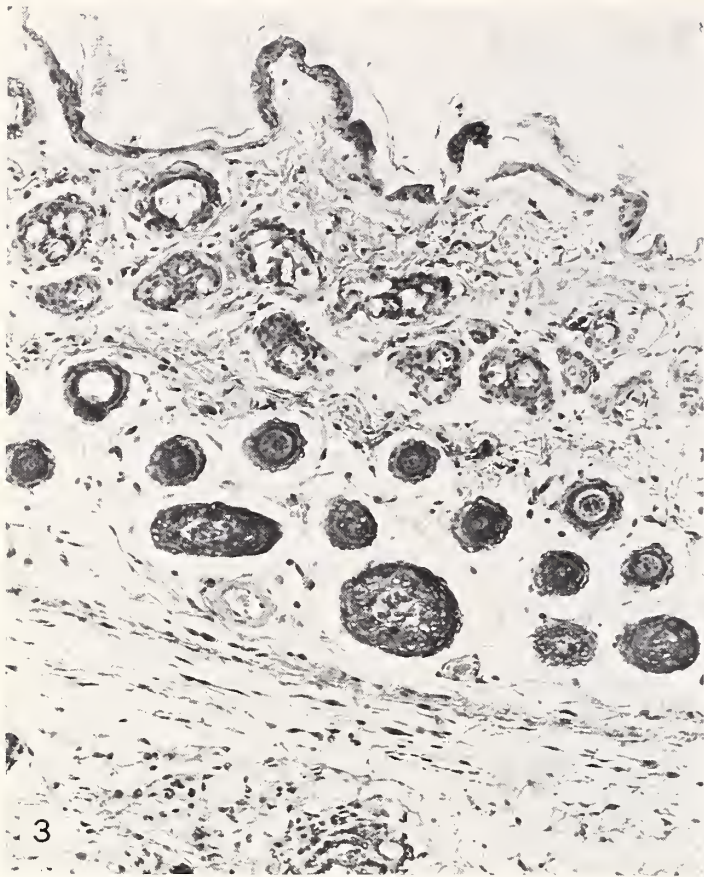


FIGURE 3.—Photomicrograph of skin taken from tumor-bearing mouse following total-body X-irradiation.  $\times 100$



FIGURE 4.—Syngeneic skin graft acceptance following GPA virus inoculation directly into newly placed graft.

## Summary: Heterogenization<sup>1</sup>

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Before we launch into our discussion, I think it would be worthwhile to summarize the historical development of the heterogenization exploration. I'll begin with a survey of the work that has been done with skin transplantation. Since Breyere and Williams conducted their investigations, many others have explored the relationship between virus and normal tissues. These investigators worked with both isografts and allografts and used diverse animal and tumor systems as indicated in table 1 of our paper on p. 310.<sup>2</sup>

Mathé, Holtermann, and Majde, and Meeker *et al.* all observed rejection of syngeneic skin taken from tumor-bearing donors and, along with Breyere and Williams and Svet-Moldavsky *et al.*, postulated virus-associated antigenic alteration of normal tissues as the basis for skin rejection. Ben-Hur *et al.* suggested that an antigenic loss caused a 7-day delay in allograft rejection. Marchant postulated that the tumor in the donor animal may have modified the antigenic properties of skin and delayed allograft rejection 1 or 2 or 4-7 days. Kaliss and Suter also postulated that the tumor in the donor animal was the causal agent. To account for the delay of 1 or 2 days in allograft rejection, they put forward 2 hypotheses: 1) Antibodies directed against tumor antigens in normal tissues can induce both accelerated and delayed rejections; or 2) immunologic enhancement mediated by autoantibodies may cause delayed rejections. McCarthy and Russfield, working with both allografts and isografts, felt that a quantitative change of some kind caused the accelerated rejection and, also, that the observed isograft rejection indicated that a qualitative change was produced in the host's tissues by the tumor. Finally, only Eichwald and Rud obtained no graft rejection.

Despite the complexities and diversities in these experiments, however, all the explanatory hypotheses advanced are closely interrelated. In effect, all investigators theorized that antigenicity of normal tissues was altered somewhat in tumor-bearing animals. Collectively, all these antigenic changes can be considered forms of heterogenization.

We had hoped that Dr. Svet-Moldavsky could have attended this Conference. But in his absence, and because of his important work in the field of heterogenization, I will summarize briefly his investigations.

The work of Svet-Moldavsky and his collaborators strongly suggests that both virus and the immune system are in some way involved in the rejection of syngeneic skin. Svet-Moldavsky *et al.* have worked with skin isografts taken from animals bearing a variety of tumors as shown in table 1.

The tumors included: 1) sarcomas induced subcutaneously with DMBA in C57BL/6J mice; 2) hemocytoblastosis La induced by X irradiation in C57BL/6J;

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<sup>1</sup> Presented at Conference on Immunology of Carcinogenesis, held by the Oak Ridge National Laboratory at Gatlinburg, Tenn., May 8-11, 1972.

<sup>2</sup> In "Tumor Cell-Related Syngeneic Skin Graft Rejection in Gross Leukemia" (Mariani T, Maruyama Y, Good RA). Natl Cancer Inst Monogr 35:309-320, 1972.



TABLE 1.—Heterogenization phenomenon: Investigations of Svet-Moldavsky *et al.*\*

Tumor or carcinogen	Tumor-bearing donor	Recipients			
		Skin graft		Tumor	
		Number rejected Total No. (%)	Days	Number developed Total No. (%)	Days
K-237 sarcoma (DMBA†-induced)	C57BL/6J	$\frac{72}{72}$ (100)	6–7 (15–20) 9–11	$\frac{8}{24}$ (34)	28–40
Hemocytoblastosis La (X-irradiated)	C57BL/6J	Not specified		$\frac{8}{8}$ (100)	7–8 (death from leukemia)
Moloney leukemia	BALB/c	Not specified		$\frac{2}{2}$ (100)	15
SB-1 sarcoma, tumor passage II (DMBA-induced)	BALB/c	None		$\frac{2}{4}$ (50)	16, 26
Hepatoma-22	C3HA	None		$\frac{3}{4}$ (75)	8
Ehrlich carcinoma	C57BL/6J	None		$\frac{8}{8}$ (100)	20–25

\* *a*) SVET-MOLDAVSKY GJ, MKHEINZE DM, LIOZNER AL: Rejection of skin grafts from tumour-bearing syngeneic donors. *Nature (Lond)* 214:693–695, 1967. *b*) MKHEIDZE DM, LIOZNER AL: Phenomenon of tumour transfer by skin grafting. *Nature (Lond)* 214:730, 1967. *c*) SVET-MOLDAVSKY GJ, MKHEINZE DM, LIOZNER AL: Two phenomena associated with skin grafting from tumor-bearing syngeneic donors. *J Natl Cancer Inst* 38:933–938, 1967. *d*) SVET-MOLDAVSKY GJ, MKHEINZE DM, LIOZNER AL, et al: Skin-heterogenizing virus. *Nature (Lond)* 217:102–104, 1968. *e*) MKHEIDZE DM, LIOZNER AL, SVET-MOLDAVSKY GJ: Tumor-induced skin heterogenization. I. Reciprocal relationship between tumors and skin grafts. *J Natl Cancer Inst* 45:465–473, 1970. *f*) SVET-MOLDAVSKY GJ, LIOZNER AL, MKHEINZE DM, et al: Tumor-induced skin heterogenization. II. Virus causing the phenomenon. *J Natl Cancer Inst* 45:475–484, 1970. *g*) LIOZNER AL, SVET-MOLDAVSKY GJ, MKHEINZE DM: Tumor-induced skin heterogenization. III. Immunologic and immunogenetic mechanisms. *J Natl Cancer Inst* 45:485–494, 1970.

† 7,12-Dimethylbenz[*a*]anthracene.

3) leukemia induced by Moloney virus in BALB/c; 4) SB-1 sarcoma induced in BALB/c; 5) Hepatoma-22 induced in C3HA; and 6) Ehrlich carcinoma passed in C57BL/6J.

Since regular rejection occurred in only one of these systems, I will concentrate on that system. Only the skin from the K-237 tumor-bearing animals (DMBA-induced sarcoma) was rejected by all 72 C57BL/6J mice, and tumor developed in 8 of 24 animals—34%. Usually by approximately 6 or 7 days after transplantation, as Svet-Moldavsky *et al.* explained in their papers, when the bandages fell off, these skin grafts had shrunk (change in graft size not indicated) and manifested necrotic foci. The recipient's skin, 15–20 days after grafting, had displaced the graft. Apparently rejection began 9–11 days after transplantation, when skin was exchanged between C57BL/6J mice bearing K-237 tumor, but the endpoint of rejection was not specified. Autotransplantation of skin in K-237 tumor-bearing mice was also performed. Auto-transplants resulted in partial rejections. But Svet-Moldavsky *et al.* did not define the criteria for partial rejections. Grafts were accepted, however, when skin was transplanted between animals that had rejected the K-237 tumor-bearing skin. Rejections were obtained in the third and fourth passages of the K-237 tumor, though no tumor developed. Apparently, skin heterogenization in this system occurred about 9 days after tumor inoculation.

To explain their observations, Svet-Moldavsky *et al.* postulated that a new antigen develops in the skin and is transferred from the tumor into the skin by 1) tumor cells;

2) lymphocytes or other cells; 3) blood, which may transfer the antigen itself or stable forms of nucleic acids, or 4) virus.

By using physicochemical and electron microscopic tests, Svet-Moldavsky *et al.* obtained further evidence that a virus may be involved in the phenomenon. The properties of the skin-heterogenizing virus (SHV) are shown in table 2, which is taken directly from their 1970 paper.

Apparently heterogenization was produced by tumor homogenates and similarly prepared homogenates of lymphoid organs, kidneys, and livers taken from tumor-bearing mice. Viable lymphoid cells also produced skin rejection. But pooled serum and brain homogenates from tumor-bearing mice did not induce heterogenization.

Several characteristics of SHV were reported. The SHV agent was inactive at 60°C—only 1 of 11 animals rejected the skin graft. It was stable after a short period at -70°C. It seems to be ether resistant. Sedimentation data indicated that SHV particles were very small. Two types of virions—175 and 80 Å in diameter—were obtained in an active fraction of the homogenate. Infectious virus was obtained from skin homogenate only once, after inoculation with tumor homogenate, but never after skin grafting.

To explore the immune-system involvement, Svet-Moldavsky *et al.* treated the recipient animals with cyclophosphamide and total-body X irradiation. At 4–10 hours before grafting, one group of recipients was total-body-irradiated with 400 R; 5 of 16 (31%) rejected the tumor-bearing skin. Another group was given injections of cyclophosphamide—200 mg/kg 24 hours before grafting and 200 mg/kg 24 hours after grafting. All 12 immunosuppressed animals accepted the tumor-bearing skin. In other words, these immunosuppressive regimens did indicate an immune system involvement.

All of the investigators I have referred to so far explored the possibility of antigenic alteration *in vivo*. Other investigators have carried out *in vitro* analyses and have obtained evidence compatible with the heterogenization concept, that an antigenic alteration or antigenic conversion of normal tissues may be induced under appropriate conditions.

By now, I'm sure that it has become obvious to you that many questions exist regarding this phenomenon. Some of these questions which need clarification follow.

1) Is our conclusion that there is no heterogenization true of other virus-induced malignancy or chemical-induced malignancy? 2) Is our conclusion of the noninvolve-

TABLE 2.—Properties of SHV

C57BL/6J donors inoculated with:	Rejection of syngeneic skin grafts in normal recipients*
Pieces of K-237 used for preparation of homogenates	24/24
Mixture of lymphoid organ cells from tumor-bearing mice	8/8
Homogenates of lymphoid organs, kidneys, and livers from tumor-bearing mice	12/12†
Homogenates of brain from tumor-bearing mice	0/4
Serum pool from tumor-bearing mice	0/4
Supernatant 1	28/32
Supernatant 1, heated for 30 minutes at 45°C	13/16
Supernatant 1, heated for 30 minutes at 60°C	1/11
Supernatant 1, frozen for 24 hours at -20°C	8/8
Supernatant 1, frozen for 24 hours at -70°C	8/8
Supernatant 1, treated with ether	8/8

\*  $\frac{\text{Number of mice rejecting grafts}}{\text{Total No. of recipients of skin grafts}}$ .

† Similar experimental results with each homogenate, with 4 mice in every group.

ment of the immune system in syngeneic graft rejection unique to the Gross passage A virus system? 3) What is present in transplanted skin taken from tumor-bearing animals—tumor cells, virus, transformed cells, or a more virulent or oncogenic agent? (Skin should also be considered a labile system that may be converted by metabolic changes accompanying tumor growth.) 4) Is the rejection of tumor-bearing skin real or is it a pseudorejection or quasirejection? (Certainly, in our system, it is not a classic rejection.) 5) Must we redefine rejection or extend the definition of rejection, and should we establish general criteria to evaluate rejection or acceptance? (We really need clarity and precision in evaluating grafting procedures.)

Finally, many of the findings on skin heterogenization are preliminary; conclusions are based on assertions or, at least, on speculations. There is a great need to introduce clarity, precision, and considerably more documentation.



## GENERAL DISCUSSION

**B. Zbar:** I have a comment and a question for Dr. Mariani. Have you ever injected X-irradiated tumor cells into skin grafts and then placed the grafts either in normal syngeneic donors or X-irradiated thymectomized donors? I believe that, in the immunosuppressed situation, unirradiated tumor cells in the graft could grow quite readily and mechanically compromise the graft. If the cells were X-irradiated, you could perhaps dissect that response and show absence of graft rejection.

**T. Mariani:** I agree that the tumor cells grow rapidly in the immunosuppressed animal. But, if they were X-irradiated before injection, they would not grow at all.

**D. J. Rubin:** I was a little disconcerted by some of the premises and comments of Dr. Martin which led to his series of experiments: one of which was the statement that EL 4 is not immunogenic in C57BL/6N mice. Briefly, I would like to summarize a series of experiments we conducted several years ago—without resorting to heterogeni-

zation or other such concepts, but going back to one of the basic principles in tumor immunology, the mode of immunization. As you know, as few as 10 cells of EL 4 kill 100% of C57BL/6N mice given intraperitoneal injections of viable tumor cells. We did a dose titration of EL 4, injected intradermally (id) or intraperitoneally (ip), as a basis for both prophylactic and ultimate immunotherapy model studies. The results are shown in table 1. EL 4 grows and regresses in the skin at doses of up to 1 million cells. The id immunization protects in a dose-dependent fashion against a graded series of ip challenge doses (table 2). Even animals that received the challenge dose of  $10^6$  cells ip after id immunization lived as long as 24 months. The specificity of these findings is shown in table 3. The way we determined the efficacy of the id injection was as follows: Only id inocula grew and regressed. If a few cells were injected subcutaneously, the tumor grew progressively and killed the animal.

TABLE 1.—Titration of EL 4 primary injections by id and ip routes in C57BL/6N mice

Number of mice	Number of cells injected	Mode	Survivors/Total
8	$10^6$	id primary	1/8*
8	$5 \times 10^4$	id primary	6/8
12	$10^4$	id primary	12/12
8	$10^3$	id primary	8/8
19	$10^2$	id primary	19/19
5	10	id primary	5/5
10	$10^7$	ip primary	0/10
10	$10^6$	ip primary	0/10
10	$10^5$	ip primary	0/10
10	$10^4$	ip primary	0/10
20	$10^3$	ip primary	0/20
20	$10^2$	ip primary	1/20
6	Medium 199	ip primary	6/6
3	$10^6$ C57BL/6N ♂ lymphocytes	id primary	3/3
3	$10^6$ C57BL/6N ♂ lymphocytes	ip primary	3/3

\* The id "takes" were confirmed by progressive growth or growth and subsequent regression of the primary inoculum.

TABLE 2.—Test for immunogenicity of id primary injection of EL 4 by ip challenge

Number of mice	id primary (No. cells)	ip challenge (No. cells)	Survivors/Total
4	$10^4$	$10^6$	1/4
4	$10^3$	$10^6$	1/4
4	$10^2$	$10^6$	0/4
6	Medium 199 or C57BL/6N lymphocytes	$10^6$	0/6
4	$10^4$	$10^4$	4/4
4	$10^3$	$10^4$	4/4
4	$10^2$	$10^4$	0/4
6	Medium 199 or C57BL/6N lymphocytes	$10^4$	0/6
4	$10^4$	$10^2$	4/4
4	$10^3$	$10^2$	4/4
4	$10^2$	$10^2$	0/4
6	Medium 199 or C57BL/6N lymphocytes	$10^2$	0/6

TABLE 3.—Specificity of id immunization with EL 4

Number of mice	id primary (No. cells)	ip challenge (No. cells)	Survivors/Total
10	10 <sup>4</sup> EL 4	2 × 10 <sup>3</sup> EL 4	10/10
9	Medium 199	2 × 10 <sup>3</sup> EL 4	0/9
10	10 <sup>4</sup> EL 4	2 × 10 <sup>4</sup> FBL*	0/10
9	Medium 199	2 × 10 <sup>4</sup> FBL	0/9
10	10 <sup>4</sup> EL 4	2 × 10 <sup>6</sup> RBL†	0/10
8	Medium 199	2 × 10 <sup>6</sup> RBL	0/8

\* Friend virus-induced ascites lymphoma induced and passaged in C57BL/6N♂ mice.

† Rauscher virus-induced ascites lymphoma induced and passaged in C57BL/6N♂ mice.

EL 4, therefore, really is immunogenic, and I think to talk about suppression of the effector cells and things like that without doing some of the obvious immunologic approaches to determine whether a cell is antigenic or immunogenic, and then suggest clinical approaches, is a little presumptuous at this point.

Incidentally, the prophylactic id immunization model works, but giving 10 cells ip in a therapeutic model does not. In animals given 100 cells ip (almost 100% lethal) and then immunized id in a sequential manner, the cells grow and regress in the skin and the mice die with ascites. I think that's the kind of result that ought to be looked at in terms of what are the effector cells that are activated by various immunization procedures and what is their distribution relative to the tumor, both of which are important determinants of the fate of a host-tumor relationship.

**W. J. Martin:** Your comments are interesting and we have not investigated whether intradermally administered EL 4 will evoke a cytotoxic lymphoid cell response. It is important to define the underlying cellular mechanisms involved in the genesis of effective immunity. Intraperitoneal immunization with Concanavalin (Con A) coated and uncoated EL 4 cells provides a useful model system with which to investigate factors regulating the development of cytotoxic lymphoid cells.

**O. A. Holtermann:** Dr. Boone, do you have any active infectious virus in your cell homogenates, and, if so, have you tried to isolate that virus and use it for immunization? I ask this question because the virus will probably be carrying host antigens (*i.e.*, tumor cell antigens) and may act as a very efficient carrier of the antigen to the immunologic system.

**C. Boone:** We have a choice between thinking in terms of virus that retains the tumor transplantation antigen on the virus envelope, or plasma membrane microvesicles of the infected cell that contain the virus hemagglutinin antigen. We favor the latter as being the most important immunogen in the homogenate.

**R. F. Barth:** Dr. Mariani, in your extensive review of the literature on heterogenization, did you come across any other histopathologic mechanisms responsible for graft rejection, or were you the first person to do this simple thing that, in your system I think, quite clearly shows that tumor cells were responsible? Also, Dr. Breyere, did you happen to study any of the grafts histopathologically and did you

see tumor cells? Finally, do you think your observations relate to the phenomenon Dr. Boone described with the influenza virus and Dr. N. P. Czajkowski described with bovine serum albumin?

**Mariani:** Regarding your question, the one thing a number of investigators did not do was to examine the skin microscopically. Many merely suggested that cells were in the transplanted skin. Our first clue came when we examined the donor skin and found tumor cells. Right from the first, we knew that we were dealing with something else in addition to the virus. We had to dissect the problem as to whether it was the virus or the tumor cells in the transplanted skin that were responsible for graft rejection.

**E. J. Breyere:** I'd like to answer that one too! Yes, we looked at the skin grafts. The tumor cell concept was not my original thought when I did it.

No, my system is not like Dr. Boone's. This relates more to the induction of antigens in the induced tumor. As Dr. Mariani describes, I have a cell that is the responsible factor.

In the skin graft rejection, though, our lymphocytic leukemia system might be somewhat of a unique system. Dr. W. Andrew has described lymphocyte migration in the epidermis; so perhaps some of these cells might actually become incorporated in epidermis. If these cells contain virus-associated antigens, these will be the effectors.

Regarding hyperplasia, characteristic of the homograft response, I don't think it is pronounced in these grafts. It may be an innocent bystander with regard to the epidermis.

**S. S. Tevethia:** Dr. Boone, what evidence have you obtained that you are dealing with simian virus 40 (SV40) tumor-specific transplantation antigen (TSTA) in your system?

**Boone:** Our evidence is the fact that animals primed with SV40-transformed cells specifically reject SV40-transformed tumors and not spontaneous or 3-methylcholanthrene (MCA)-induced tumors. The SV40 tumors we use are positive for T antigen, of course.

**I. P. Witz:** Dr. Martin, did you obtain any evidence of humoral immunity after immunization with its Con A-coated tumor cells?

**Martin:** We have not looked extensively for antibody production in EL 4-immunized mice. Low levels of cytotoxic antibody have been detected in both EL 4- and Con A-

coated EL 4-immunized mice. This study needs to be extended, and at present we do not know if significant differences in antibody production exist between the groups. The report of Cohen and Feldman demonstrating the *in vitro* generation of autoimmune lymphoid cells suggests to us that the mechanism restricting the *in vivo* development of antitumor cytotoxic lymphoid cells may primarily be concerned with the suppression of autoimmunity. The possibility of evoking autoimmunity as a complication of tumor immunization, either *in vivo* or *in vitro*, must be borne in mind.

**W. E. Paul:** I would like to raise a point concerning the fact that you found the Con A coated to different tumor cells had an effect very similar to that seen with Con A coated to EL 4 cells, but free Con A was ineffective. Several articles have appeared recently reporting that Con A on solid surfaces may well stimulate B-lymphocytes, although Con A in the free form preferentially activates T-cells. Moreover, some preliminary work suggests that Con A suppresses the production of antibodies *in vitro*, even when it is on solid surfaces.

Is there any way you can incorporate these considerations into your view of what effect Con A has on the immune system?

**Martin:** It is difficult to assign a role for Con A when we know so little of the cellular events involved in the generation of cytotoxic lymphoid cells. The experiment involving Con A-coated IM 9 cells given together with uncoated EL 4 cells supports the concept that Con A may act directly on the immune system. In the experiment, however, both the Con A-coated IM 9 cells and the uncoated EL 4 cells were administered intraperitoneally. Possibly an exchange of Con A between the 2 cell types may have occurred.

**M. A. Basombrío:** In some of the experiments of Svet-Moldavsky, nononcogenic viruses (I think vaccinia or influenza) were used to heterogenize skin or tumor cells. Evidently no new tumor cells were produced in the grafts. Dr. Mariani, could you explain how these rejections occurred?

**Mariani:** I don't know. As far as the virus postulate of Svet-Moldavsky, careful reading of his papers indicates that, while he shows that the virus is there, he does not say it is an oncogenic virus. Rather he suggests that it may be a passenger virus or perhaps a new group of oncogenes. I can't interpret any more than that from his material.

**Zbar:** Dr. Martin, have you challenged the animals immunized with Con A-treated EL 4 cells or X-irradiated EL 4 cells, and have you seen any protection against challenge with viable EL 4 cells?

**Martin:** We have just completed a small survival study. Mice immunized optimally with Con A-coated EL 4 followed by uncoated EL 4 are still susceptible to an intraperitoneal challenge of live EL 4 but show a significantly prolonged survival time. Immunized mice inoculated with  $10^7$  live EL 4 survive approximately the same time as non-immunized mice inoculated with  $10^6$  live EL 4.

**D. Axler:** I would like to comment on questions directed to Dr. Boone. As Dr. Boone mentioned, we prepared Newcastle disease virus (NDV) lysates of SV40-transformed cells and have induced tumor immunity.

The question from Buffalo centered around: Is the antigen

associated with the virus, or is it separate? With sucrose-density separation procedures, we can separate the virus. The antigen appears to be distinct from the virus, so it is not part of the viral envelope. Evidently it is released by virus-cell interaction.

Regarding Dr. Tevethia's question, as to how do you know it has the SV40 TSTA, with the NDV system, when we made NDV lysates of cell lines transformed by agents other than SV40, or by normal cells, we could not induce immunity.

**Boone:** As to whether it is a virus or a plasma membrane microvesicle, it's surprising how similar these 2 structures are. A plasma-membrane microvesicle is about the size of an influenza virus and has a similar density. It frequently encloses RNA in the form of ribosomes and has the viral hemagglutinin and the neuraminidase antigens on its surface. It will absorb to red cells and agglutinate just like the virus. So it is very difficult to separate one from the other. Since there is more plasma membrane than there is virus envelope, we vote for the postulate that the virus-modified plasma-membrane microvesicles are doing the immunizing.

**Rubin:** Just a brief comment. What I really would suggest is that you not use EL 4 for these studies, because of one other finding we made on this tumor cell line. Although the cell line you used may be different—EL 4 has a very complex history—it has lost surface antigens.

Using the C<sub>I</sub> fixation and transfer test that Dr. Leonard mentioned to detect virus-specific antigens and antibodies with EL 4 as the "control" for extensive absorption, this is what we found with heterologous anti-Rauscher serum.<sup>1</sup> There were inhibition and then apparent enhancement of C<sub>I</sub> fixation with EL 4. Finally, after 10 or 12 absorptions of the serum with EL 4, there was significant residual reactivity against either Friend or Rauscher virus-induced tumors. At this point you might say, "Ah, ha! I've got the virus-specific antigen and antibody" for further study. However, when we tested the absorbed serum against normal C57BL lymphocytes, we detected significant complement fixation.<sup>2</sup>

**Martin:** There are advantages and disadvantages to studying long-term tumor lines such as EL 4. These tumors doubtless undergo mutational changes and are contaminated by a variety of viruses. EL 4 maintained in different laboratories may express different tumor-associated antigens and what may apply to one tumor line may not be generally true. Nevertheless, these are valuable tumors with which to define clearly different immune mechanisms. It is then possible to investigate whether these mechanisms operate in autochthonous tumor-bearing hosts.

**M. M. Bortin:** Dr. Mariani, is there anything in your data, or have you done any experiments, to exclude the possibility that the leukemic infiltrate rejects the skin graft? In mixed leukocyte culture, the AKR leukemic cells can react against normal AKR cells, which have been treated with mitomycin.

<sup>1</sup> See also text-fig. 3, p. 978. In RUBIN DJ, COLTEN HR, BORSOS T, et al: Antigenic loss in a transplantable, chemically induced leukemia of C57BL/6 mice. J Natl Cancer Inst 44:975-979, 1970.

<sup>2</sup> See also table 1, p. 979, of above article.



**Mariani:** No, I haven't done any studies along those lines as yet.

**A. J. Girardi:** In the immunosuppressed animals, did you ever put in skin that had been irradiated?

**Mariani:** No, we transplanted tumor-bearing skin onto the immunosuppressed animal.

**Girardi:** Did you have rejection?

**Mariani:** Yes.

**Girardi:** If skin had been irradiated, would you hypothesize that the skin graft would not be rejected?

**Mariani:** Yes, the skin graft would not be rejected.

**Boone:** Did you X-irradiate the skin that you transferred?

**Mariani:** Oh, yes. That was the crux of the second major series of experiments. We total-body-irradiated the donor before skin transplantation. We showed quite effectively that, as you decrease the number of tumor cells in the skin, both the rejection rate and the incidence of graft-site tumor go down. That result clearly showed, just like with isolated lymphoma cells irradiated *in vitro*, that, if you delay the tumor onset or the development of the tumor, tumor-bearing skin will be accepted.

**Holtermann:** I'm another one of the very unfortunate workers in the skin transplantation field! We worked with the lymphocytic choriomeningitis virus system in mice. Normal mice will consistently reject skins from chronic virus-carrying animals.

Dr. Breyere, did you ever look for any antigenic alterations in normal cells in the skin of your infected animals by fluorescent microscopy or by any other method?

**Breyere:** No, we have not.

**M. M. Sigel:** Dr. Martin, have you noticed any increase of blastogenic transformation with Con A in the lymphocytes of animals immunized with EL 4 cells coated with Con A?

**Martin:** We have not looked for increased blastogenesis in the spleens of mice immunized with Con A-coated EL 4. Splenomegaly occurs in mice given injections of both Con A-coated and uncoated EL 4.

**Sigel:** Dr. Boone, have you tried injecting influenza virus separately from the tumor antigen to see whether there was a potentiating effect?

**Boone:** We did something very similar to that. We mixed the influenza virus grown in eggs with the tumor homogenate, and this appeared to have little, if any, protective effect.

**B. Bonavida:** Dr. Martin, have you used in your system any substance other than Con A to increase the response to EL 4 cells?

**Martin:** In our initial studies we attached dinitrophenyl amino caproate to EL 4 and observed a slight increase in immunogenicity.

**Bonavida:** How about a protein or a high-molecular-weight substance, *e.g.*, bovine serum albumin or gamma globulin?

**Martin:** We have not tried to attach macromolecules, other than Con A, to EL 4 cells.

**Bonavida:** We did some studies in which we coupled gamma globulin to the EL 4 cells and we also saw a prolongation of the survival of the animal when challenged with the live tumor cells.

**D. Steinmuller:** Dr. Mariani, I would like to propose an experiment to test Dr. Bortin's interpretation of your results. What would happen if you thymectomized and sublethally X-irradiated an F<sub>1</sub> hybrid, grafted skin from a normal parent strain mouse onto that hybrid, and 24 hours later, injected sensitized cells from the other parent strain into the skin? In other words, you would have a piece of skin on a hybrid containing allogeneic cells reacting against those skin components.

**Mariani:** This system has been so unusual that I'm not going to speculate what would happen in that type of experiment.

**Breyere:** Dr. Steinmuller, I haven't done exactly that experiment. But we have taken skin from a BALB/c mouse and transplanted it onto a histocompatible F<sub>1</sub> hybrid. After 100 days, the skin was retransplanted back to the strain of parent origin. Many of these grafts were rejected as a result of acquiring antigens contained in the hybrid host.

**Zbar:** I have one comment about procedures of attempting to increase immunogenicity in tumors. It seems to me that weakly antigenic, syngeneic tumors must be studied by the procedures discussed, as well as by other procedures. If one takes a strongly antigenic SV40-induced tumor, with which it is relatively easy to induce long transplantation immunity by customary procedures, then the study of the influenza approach doesn't really answer the question of whether you can augment the immunogenicity of a weakly antigenic tumor as would be seen in the spontaneous host. This is where these methods need to be critically tested, *i.e.*, in tumors, which, when tested by usual criteria (*e.g.*, transplantation protection), do not immunize or do so exceedingly weakly.

**Boone:** I agree profoundly with Dr. Zbar's comment. We plan to test next some MCA-induced tumors, in which we have already found a certain immunogenic effect with the influenza virus, some mammary tumors of mice, and some squamous cell carcinomas induced by chemicals in mice.

**Paul:** I commend the audience and also our panelists. I will not attempt to draw the consensus of the group at this time.

## **SESSION 7**

### **Diagnosis and Immunotherapy of Cancer**

**Chairmen: Edmund Klein and P. Burtin**





## Introduction: Diagnosis and Immunotherapy of Cancer<sup>1</sup>

Edmund Klein, M.D., Chief, Department of Dermatology, Roswell Park Memorial Institute, Buffalo, New York 14203

Immunologic approaches to problems in tumor biology and to the possible treatment, diagnosis, and prevention of malignant diseases go back to the turn of the century. The presence of tumor-specific antigens in animal tumors has been well documented, and a substantial amount of evidence for the existence of such antigens in tumors of man is accumulating. The role of immune factors in controlling neoplastic growth has been established in animals. Increased susceptibility of neonatally thymectomized animals to chemical carcinogenesis or tumor viruses has been demonstrated by several workers. Since a noticeable effect of neonatal thymectomy is a reduced capacity to reject homografts, the increased and accelerated occurrence of neoplasms in thymectomized animals is likely to be related to a reduced ability to inhibit or destroy potentially malignant cells.

Various vaccination procedures have been shown to be effective in suppressing or preventing neoplastic growth. Working with tumor induction by simian virus 40 (SV40) or adenovirus 12 in hamsters, B. E. Eddy and her co-workers could protect animals that had been infected with the virus as neonates against tumor induction by injecting them with repeated doses of the respective viruses after they had reached immunologic maturity. More directly, H. Goldner could interrupt tumorigenesis with SV40 in hamsters by intraperitoneal injection of syngenic non-virus-producing SV40-transformed cells that had been rendered nonviable by X irradiation. Similar findings were reported by A. J. Girardi, also in hamsters, using viable human cells transformed by SV40 *in vitro* rather than X-irradiated hamster cells.

The immune mechanism responsible for graft rejection is intimately associated with lymphoid cells and is related to delayed-type hypersensitivity. Most likely, a similar mechanism is also involved in the control of neoplastic growth. L. J. Old and co-workers found that the level of induced immunity may be sufficient for rejecting small numbers of grafted tumor cells while larger inocula resulted in tumor take. G. Klein and associates observed that tumor cells, treated *in vitro* with leukocytes obtained from hosts sensitized against the tumor, had lost their ability to grow after transplantation into syngenic hosts. These observations indicated that specific cell-mediated immunity was an important factor in the relation between the host and tumor.

P. Alexander and his group as well as Old and co-workers demonstrated that adoptive immunity transferred protection from tumor-bearing animals to syngeneic recipients of tumor transplants. These studies, emphasizing the role of tumor-specific transplantation antigens and the resulting specific cell-mediated immunity, were complemented by the studies of D. W. Weiss and of E. A. Boyse showing that non-specific stimulation of cell-mediated immune reactivity also increased protection against virus-induced tumors as well as transplanted tumors in syngenic animals. Thus it became apparent that induction of sensitivity to bacille Calmette-Guérin

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<sup>1</sup> Presented at Conference on Immunology of Carcinogenesis, held by the Oak Ridge National Laboratory at Gatlinburg, Tenn., May 8-11, 1972.

(BCG) increased the responsiveness of the immune system against tumor cells, preventing their further development.

These principles were subsequently found to extend from tumor prevention to the induction of regressions and eradications of established malignant lesions in man and in animals. H. J. Rapp, B. Zbar, T. Borsos, and their co-workers showed that induction of immunity to BCG at the site of tumor implantation not only prevented the development of tumors but also resulted in regression of tumors which had become established. They showed, furthermore, that lasting regressions included metastatic lesions in the regional lymph nodes without direct administration of BCG to the nodes involved by tumor. The guinea pig model system used by these workers has provided us with a means to study in detail these parameters which cannot be adequately assessed in humans. Further studies on this model will be discussed in this session by Zbar and M. G. Hanna to bring us up to date on the current status in this area.

After vaccination with tumor cells treated with neuraminidase, tumor regressions apparently based on specific immunity against tumor antigens have been reported by Simmons and co-workers. The data indicate that changes induced on the surfaces of the tumor cell result in a more intense response in the efferent and possibly also in the afferent arm of the immune mechanism. It is noteworthy that L. Weiss has shown that treatment of lymphocytes with neuraminidase also results in a more intense reaction against untreated tumor targets. L. Warren, who has extensively studied sialic acid complexes on cell surfaces, found that the sialic acid moiety and the conformation of the associated proteins differ markedly between normal cells in their resting stage and cells undergoing reduplication. In normal cells, this alteration in the sialic acid content is limited to the period of active cell division. In tumor cells, a larger sialic acid component of surface glycoproteins appears to be present even when the cells are not dividing. Warren further found that sialic acid complexes appear to inhibit protein-protein interactions. Thus the sialic acid protein complex is apparently related to significant changes on cell surfaces as they undergo interaction and possibly is not limited to immunologic mechanisms. The contributions of cell biology as presented at this Conference during the past 3 days clearly are of major significance, whether to our understanding of antigens on the cell surface, to the unmasking of obscured antigens, or to the induction of new antigens. One would hope that the action of neuraminidase is but one example of other possibilities for increasing the intensity of cytotoxic effects of immunologic reactions on neoplastic cells.

The experience in immunotherapeutic approaches in animals, which I have briefly outlined, is paralleled to some extent in human oncology. Thus both tumor-specific immunity and non-(tumor)-specific immunity have been shown to inhibit tumor development and growth by preventing its onset, to retard or arrest the further development of established tumors, or to induce eradication without residual or recurrent tumor during prolonged observation periods. The implications of the findings in animals on the effects of immunotherapeutic approaches to human cancers are limited by the nature of some of the experimental tumors which were studied. It is generally accepted that virus-induced tumors and transplantable tumors in syngeneic hosts have common tumor antigens, while the antigenic profiles of carcinogen-induced tumors vary considerably as shown by R. T. Prehn and associates. Since malignant tumors in man may resemble more closely the variability in the antigenic profiles of carcinogen-induced tumors than the more homogeneous virus-induced or transplantable animal tumors, caution should be exerted in direct extrapolation of the observations from the latter experimental tumor types to cancer

in man. On the other hand, even the initial antigenic homogeneity of virus-induced or transplantable tumors is subject to neoantigen formation and antigen deletion as selection of tumor growth against immune resistance takes place. One may suspect that in this respect there is some resemblance between experimental tumors of viral or transplanted origins and the antigenic components of human cancer as it metastasizes or progresses from multifocal sources. Conversely, despite the generally accepted variability of the antigenic composition of carcinogen-induced tumors, the possibility of underlying common structural or conformational properties of tumor antigens may be of significance. In this respect again, there may be some similarity between chemically induced animal tumors and human neoplasms.

The status of immunotherapeutic approaches to cancer in man parallels the experience in animals with a number of important reservations. Both tumor-specific immunity and non-(tumor)-specific immunity have been explored in human cancer. The differentiation between tumor-specific immunity and nonspecific immunity is arbitrary and primarily refers to the manner in which immune processes directed against tumors are initiated. We know too little about the mechanisms to decide whether there is a final common path by which induction of immunity, utilizing antigens other than those of tumor origin, may ultimately involve interaction with tumor-specific antigens.

The use of nonspecific immunity in the prevention of the development of malignant lesions in man apparently is possible. Our observations in patients with syndromes characterized by a persistent or increasing rate of development of multiple, new, neoplastic lesions indicate that nonspecific immunotherapy markedly reduces or completely eliminates the development of new tumors. Studies in collaboration with R. Herberman are oriented toward determining whether a common tumor antigen in multifocal epidermal tumor syndromes (*e.g.*, xeroderma pigmentosum, multiple basal cell carcinoma) can be demonstrated. Our findings in premalignant lesions of epidermal origin, such as leukoplakia involving the mucous or mucocutaneous structures and precancerous keratoses of the epidermis, indicate that nonspecific immunotherapy results in their eradication, thus preventing progression to the frankly malignant stage. We do not know whether we are actually preventing the development of malignant cells or eradicating them almost as rapidly as they are formed, thus preventing progression to clinically manifest cancer.

The syndromes characterized by the persistent development of multiple malignant lesions permitted us both concurrently and, more importantly, sequentially to assess in man the feasibility and potential effectiveness of interfering with the development of malignant neoplasms by immunologic or other means. Unfortunately, with the possible exception of isolated experiences with chemotherapy, other therapeutic modalities such as ionizing radiation, hormone therapy, cytotoxic chemotherapy in general, and surgery have not reduced the incidence of tumors. Studies in patients with multiple tumor syndromes may indicate the feasibility of developing a vaccine for the prevention of malignant diseases within a relatively short time, as contrasted to a vaccine against solitary malignant diseases which will require a considerably longer time to explore, develop, and evaluate.

Regression of malignant lesions in man induced by immunotherapeutic approaches of both tumor-specific and nonspecific nature has been demonstrated. Eradication of malignant lesions after the induction of the cell-mediated immune challenge reactions at tumor sites was demonstrated in patients with epidermal cancers and precancerous epidermal lesions.

While epidermal neoplasms facilitate immune approaches to provide definitive results in terms of regression, eradication, partial or temporary control, and recur-



rences, the extension of the underlying principles to malignant disease in general will require further investigation. However, considerable progress has been made in exploring the feasibility of utilizing both tumor-specific and nonspecific immunity for the induction of regressions of disseminated malignant diseases. The presentations to be made in this session by G. Mathé, D. L. Morton, and L. J. Humphrey, as well as by our group, are concerned with this subject. Studies by Sokal and his associates further suggest that nonspecific and, possibly, tumor-specific immunity may be of significance in the management of chronic myelogenous leukemia. The study recently reported by R. Siegler and his group on melanoma also indicates that both tumor-specific and nonspecific immunity may be effective in immunotherapeutic approaches to melanoma. Thus, immunotherapy of human cancer is still in the early exploratory stages. The feasibility of immunologic approaches to the management of malignant disease has generally been accepted as a basis for intensified efforts in that direction. Further systematic progress in this area will depend to a large extent on understanding the mechanism underlying those immunologic approaches to the control of cancer which have not been effective or may have contributed to tumor progression.

The essential components of the interactions between the immune system and tumors include tumor-specific antigens, humoral antibodies, and/or cell-mediated immune mechanisms. The cell-mediated system, including specifically sensitized lymphocytes capable of direct interaction with the target antigen and macrophages which may or may not intervene in this reaction, appears to be a primary effector component. The humoral antibodies against tumor antigens may be blocking or enhancing, thus presenting an obstacle to the antitumor action of the cell-mediated immune system. An understanding of the interrelation of apparently opposing immune factors is therefore of great importance. Further attention should also be given to cytotoxic humoral antibodies which may act either directly or indirectly as cytophilic antibodies.

In the vast majority of established tumors, the host's spontaneous defense mechanisms or natural immune surveillance system appears to be inadequate. One prime objective of immunotherapeutic approaches, therefore, is to induce immunity against tumors which will destroy malignant cells in the otherwise immunocompetent individual. In the patient with impaired immunocompetence, methods for re-establishing immunity would be required. Usually impairment of immune competence covers a spectrum, both quantitatively and qualitatively, ranging from a normal state (which cannot be adequately defined) to a complete loss of immune responsiveness. Furthermore, immuno-incompetence may involve one or only a few antigens and the patient may be fully competent as far as other antigens are concerned. Apparently the cell-mediated system, at least in malignant disease, is more vulnerable than the humoral antibody system. Thus the balance between tumor inhibition by the cell-mediated system and blocking effects of humoral antibodies may be disturbed in favor of protecting or enhancing tumor growth. Induction of passive immunity by administration of immunocompetent lymphocytes is not a practical procedure, since the viability of allogenic lymphocytes is limited either due to rejection by the host or to the inherent instability of such cells; also, the quantity of the lymphocytes which would be required is difficult to obtain. However, there is some hope that active immunity might be induced by transfer by intact lymphoid cells or a transfer factor. It has been reported that cell-mediated immunity against microbial antigens and a number of small organic molecules (haptens) can be transferred in man. Whether immunity against tumor antigens can be transferred has not been resolved but appears at least feasible. Local passive transfer of immunity has also been demonstrated in animals and man by administration of allogenic lymphocytes. The possi-

bility of using local passive transfer by intralesional administration of lymphocytes into accessible tumors has been explored.

The induction of passive immunity by administration of humoral antibodies has been attempted. Cytotoxic species of antibodies need to be isolated and prepared in relatively pure form to exclude the presence of blocking antibodies. Studies on melanoma, sarcoma, leukemia, clear cell carcinoma of the kidney, and Burkitt's lymphoma suggests that this approach may be feasible.

Adoptive immunity, which has been used in studies on experimental tumors, is not a practical procedure in humans, since only identical twins present an indication which would parallel animal studies. Studies on adoptive immunity in humans were carried out for the primary purpose of eliciting a specific antitumor immunity response, but have not been conclusive. Investigations of bone marrow transplants between twins, one of whom had leukemia, included exploration of effects on the immune systems. Transmission of immune responsiveness from the donor to the recipient was suggested. Since sex markers were not available, it was not possible to conclude whether the induction of immunity to a given antigen in the recipient was due to adoption of immunity or to transfer of immunity. In the same studies, however, humoral antibody activities previously not present in the recipient became apparent after bone marrow transplantation. This would suggest that at least the cells responsible for the formation of humoral antibodies had been adopted.

Antitumor activity of the cell-mediated system may be due to at least one of the following 3 types of interaction: It may be specific for the tumor antigen; it may have selective action against tumor cells without being immunologically specific; or it may be a nonspecific effect as exerted by aggressive macrophages. Possibly all 3 types of activity are involved to varying degrees in addition to other factors as yet unsuspected. Establishment of a tumor-specific activity on the part of lymphocytes for therapeutic purposes may be facilitated by the isolation, characterization, and identification of specific tumor antigens. Presumptive indications to that effect are provided by the studies on interactions of lymphocytes with tumor cells which suggest that tumor cells are specifically destroyed without concomitant adverse effects on non-malignant cells. Selective action by macrophages (in addition to classic immunologic specificity) in a final common path for tumor cell destruction following an initial immune stimulus is suggested by studies of O. Holtermann of our laboratories. The data indicate that aggressive macrophages may act through a recognition mechanism which can be altered by serum factors, thus increasing or decreasing the cytotoxic activity of macrophages. Whether the serum factors are immunoglobulins or some other discriminative agent is not known at present. Parallel studies by Holtermann using purified protein derivative (PPD) in tuberculin-positive patients with mycosis fungoides have resulted in tumor regressions at the site of delayed-hypersensitivity reactions as well as distant sites which had not been challenged with PPD. Similar observations were made on the effects of a number of sensitizing agents ranging from organic molecules like dinitrochlorobenzene (DNCB) to complex antigenic systems like BCG.

Tumor-specific and nonspecific immune stimulation has been attempted to increase the level of antitumor activity of the cell-mediated system. Nonspecific immunity has been augmented by primary induction of immunity and challenge with a number of haptens, as well as the use of immune memory for a number of microbial antigens (*e.g.*, PPD). Intact microorganisms have also been investigated; among these, BCG was found to be more effective than most other agents used. Whether this is due to a unique property of intact BCG or a component located in the cell wall has not been established.

Stimulation of immunity directed against tumors may be complicated by induction of blocking or enhancing antibodies. Further understanding of cytotoxic antibodies, immunoglobulins which may participate in a cell-mediated reaction, cytophilic antibodies which may contribute to the selectivity of aggressive macrophages for tumor cells, or of a de-blocking phenomenon is clearly of major importance to progress in clinical immunotherapy. Progress in this area is indicated by the encouraging therapeutic effects of cytotoxic humoral antibodies against malignant cells reported in studies on sarcoma.

The mechanism by which nonspecific immunity induces antitumor effects is almost completely unresolved. Several possibilities have been suggested. There may be a final common path which results in tumor destruction, probably predominantly mediated by selective activity of "aggressive" macrophages. The action of small, organic molecules acting as haptens, at least in the initial phases of eliciting cell-mediated immunologic response, may differ from that of microbial antigens. Whether haptens like DNCB combine with one or more of the numerous proteins to which they may complex and whether one or more of these proteins are surface components of tumor cells which qualitatively or quantitatively differ from normal cells have not been clarified. It is unlikely that microbial antigens would directly interact with tumor antigens or convert nonantigenic components of the tumor cell surface to antigenic target sites. In any event, cell-mediated challenge responses, whether induced by hapten complexes or by discrete antigenic microbial agents, result in an accumulation of mononuclear cells. As a result, macrophages are activated which then may proceed to destroy the tumor cells. Another effect of this reaction may be the processing of tumor antigens by macrophages resulting in a stimulation of tumor-specific immunity.

It has been observed that 2 or more antigens can produce a challenge reaction when single antigens have failed to do so. It is therefore possible that the presence of a potent antigen acts as an adjuvant in the induction of effective immune responses to an otherwise weak tumor antigen. The presence of a relatively large number of lymphocytes could also increase the likelihood of sensitization of these cells to a tumor antigen. If this were to occur *in situ* in the presence of a notoriously labile tumor antigen, it might explain the more intense challenge reaction by lymphocytes accumulated at the site of tumors than in normal tissue. Thus, accumulation of lymphocytes, induced as a result of challenge to a strong antigen, may place them in a strategic position to develop sensitivity against a tumor antigen with which their contact would otherwise be more limited.

The role played by noncellular mediators in the antitumor activity of the cell-mediated challenge reaction presents an intriguing problem which may be of great significance in theoretical considerations as well as in practical approaches to immunotherapeutic management of malignant disease. The various noncellular mediators released during cell-mediated immune challenge are currently under study but, except for macrophage migration-inhibiting factor, relatively little is known about these factors and further investigation is urgently needed before serious consideration can be given to clinical implications.

More complex aspects of the mechanisms would involve the induction of T- to T-cell interactions or T- to B-cell interactions as a result of the induction of 2 or more antigens at the site of neoplastic lesions, thus inducing a selective antitumor response.

To extend the effectiveness of immunotherapy for cancer, the following aspects require understanding and clinical implementation: 1) intensification of immune mechanisms which selectively destroy tumors and prevent their development; 2) control of blocking antibodies and other obstacles against cytotoxic immune effects on tumor cells; and 3) repair of immuno-incompetence in patients in whom it is defective.



To assess and further develop tumor-specific immune reactions, the demonstration and subsequent preparation of purified tumor antigens obtained from human cancers appear to be prerequisites. Purified preparations of tumor antigens would facilitate quantitative assessment of the interactions of immune factors and may provide a means for manipulating the immune response.

Immunoglobulins which may block or promote cytotoxic immune effects must be characterized. Availability of purified tumor antigen may considerably facilitate the quantitative determination of circulating levels of blocking antibodies. Tumor antigen may furthermore permit the purification and eventual clinical control of blocking and of cytotoxic antitumor antibodies. Further information in this area may also be of importance in exploring the possibility of passive serotherapy of cancer.

Immunocompetence is frequently impaired in patients with malignant disease. The information available indicates that some form of depression of immunocompetence may have preceded the onset of tumor development. Nevertheless, apparently normal degrees of immunocompetence can be demonstrated in patients with established and progressive malignant disease. While a cause-and-effect relationship has not been proved, correlation between progression of disease and immunocompetence has been reported by F. R. Eilber and Morton as well as by others.

Data obtained in our clinical studies suggest that the ability to develop immunity to antigens to which the host has not previously been exposed is decreased in the initial stages of impairment of immunocompetence associated with malignant disease. Some studies by others confirm these findings, whereas those of other workers failed to show this as a constant phenomenon. Our studies show that a significant number of patients who fail to develop immunity to sensitizing agents to which they are newly exposed retain immunologic memory from previous immune experiences—notably to microbial antigens—to which the patient had been exposed before the onset of malignancy.

While the clinical objectives of tumor immunology are reasonably well recognized, progress will depend on the current state of research in those areas in which results bearing on immune therapeutic and diagnostic advances may be expected.

Purification of tumor-specific antigens by modifications of Reisfeld's method for the separation of transplantation antigens has shown promise. Rapp and his associates, by a modification of Reisfeld's method, could prepare fractions containing tumor antigens from the hepatoma which had been used in their guinea pig model system. Herberman is pursuing fractionation methods for obtaining preparations of human tumor antigens.

Considerable progress has been made in studies on differences in the surfaces of malignant and normal cells. Studies by Burger indicate that components of cell surfaces can be manipulated without loss of cellular integrity. His studies suggest that subtle changes in the properties of the cell surface have important effects on the characteristics of tumor cells. The studies of Warren, previously referred to, show differences in the conformational and compositional characteristics of sialic acid containing glycoproteins on the surfaces of tumor and normal cells.

Studies by Manson on separation of a lipoprotein fraction containing tumor-specific antigens may be of significance for diagnostic or therapeutic approaches. The large volume of work currently in progress on characterizing and separating tumor antigens in animal tumors is likely to provide important information for analogous studies on human tumor material.

Hopes for vaccination against tumor development in man will depend on these studies of the characteristics of tumor antigens, their relationship to each other, and the feasibility of controlling the development of blocking or other antibodies which may interfere with cytotoxic actions of the immune system.

One major obstacle to effective tumor immunotherapy may be blocking antibodies as reported by K. E. and I. Hellström. Various approaches, such as deblocking or saturation of circulating antibodies with tumor-specific antigens, are being explored. The latter approach requires careful consideration of the possibility of inducing an anamnestic response, thus further raising the levels of blocking antibodies as a result of an attempt to neutralize them with tumor-specific antigen.

Current studies by C. McKhann on controlling levels of antibodies by suppressing plasma cell activity by means of antibodies against plasma cells may have bearing on immunotherapy. Another approach currently under investigation is the extracorporeal removal of blocking antibodies by removal of immunoglobulins through thoracic-duct drainage. There are a number of other possible avenues for extracorporeal removal of immunoglobulins by specific absorption techniques.

Impairment of immunocompetence in malignant disease is a serious problem to which there are few if any promising remedies. Removal of a large proportion of malignant tissue has been reported to be followed by lowering of blocking antibody levels and an increase in cell-mediated immune responsiveness. It appears that current methods by which tumors are removed by surgery, chemotherapy, or radiation therapy may have significant bearing on the state of immunocompetence. These considerations may pertain significantly to the effectiveness of immunotherapeutic approaches.

Studies on adrenalectomy in patients with carcinoma of the breast and other endocrine-dependent tumors are of significance in regard to increasing immunocompetence of the cell-mediated systems by removing endogenous steroids which may be acting as immunosuppressive agents. In this respect, chemical agents which suppress adrenocortical activity may be of interest.

An important consideration in regard to the impaired immunocompetence in malignant disease is the action of chemotherapeutic anticancer agents, most of which are immunosuppressive. Recently developed methods in which high doses of chemotherapeutic agents are used in a pulsed fashion at protracted intervals, particularly in association with such a system as the methotrexate citrovorum system of I. Djerassi, may permit recovery of the immune system between successive chemotherapeutic treatments and reduce the depression of the immunologic mechanisms.

In addition to reversing impairment of immunocompetence, enhancement of immune responsiveness of the cell-mediated system is clearly indicated.

Induction of neoantigens by heterogenization as studied by C. W. Boone offers an avenue for immunotherapeutic explorations. Various other approaches including tumor-specific antigens and nonspecific immunity have already been considered. One of the most potent stimulants of cell-mediated immunity, the transplantation antigens, deserves consideration in this respect.

Transfer of the immunity, active, passive, or adoptive, has also been considered. In this connection, the possibility of graft-versus-host reaction requires attention. There are some recent indications that partial control of a graft-versus-host reaction may be feasible and that the antigenic responses may be directed preferentially against the tumor rather than the host.

Finally, neoplasia should be recognized as a disturbance in homeostatic mechanisms which evolutionally precede the development of a complex, delicately balanced system as seen in advanced species like man. Before the advent of an exquisitely sensitive immune system, recognition mechanisms were present in more primitive forms which provided defense mechanisms against agents that threatened the identity of the organism. These primitive recognition mechanisms operate at lower levels in the phylogenetic scale against microorganisms and a variety of agents which would

be regarded as antigenic by more advanced species. It may be worth considering the possibility that the development of neoplasia in more primitive species was under the control of the same primitive recognition mechanisms. It is possible, therefore, that some of these primitive recognition mechanisms may not have been forgotten by more advanced species. In addition to the specific activities of the immune system, the possibility should be kept in mind that these more general and more primitive recognition mechanisms may be helpful in understanding and augmenting the defense against malignant disease.





# Tumor Regression Mediated by *Mycobacterium bovis* (Strain BCG)<sup>1</sup>

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**SUMMARY**—1) *Mycobacterium bovis* (BCG)-mediated tumor killing requires a host that can develop and express an immune reaction of the delayed-hypersensitivity type. 2) Effective mycobacterial preparations cause the development of cellular immunity to BCG antigens and persist in the tissues of the host to elicit delayed-sensitivity reactions. 3) BCG-mediated tumor killing requires BCG at the tumor site. 4) BCG-mediated tumor regression requires an adequate dose of living BCG. 5) BCG-mediated tumor killing requires the development of a chronic inflammatory reaction of the delayed-hypersensitivity type at the tumor site. 6) BCG-mediated tumor regression is limited by tumor size. 7) BCG-mediated tumor killing may occur without the development of systemic specific tumor immunity. 8) Specific antitumor immunity may develop from BCG-mediated tumor killing. 9) BCG cell walls attached to oil droplets have potent tumor-suppressive properties. 10) Tumor cells are killed at sites of BCG infection as "innocent bystanders." 11) Tumor cells are probably destroyed by activated macrophages. 12) BCG-mediated tumor cell killing may lead to the development of augmented specific tumor immunity.—*Natl Cancer Inst Monogr* 35: 341-344, 1972.

UNDOUBTEDLY *Mycobacterium bovis* (BCG) infection inhibits tumor growth (1, 2). But it has been difficult to harness this potential therapeutic effect of BCG for the patient with cancer. The present report discusses the results of experiments to elucidate the cellular and molecular basis of BCG-mediated tumor killing. Improved understanding may lead to more effective use of BCG in cancer therapy.

Possibly inhibition of tumor growth by BCG does not require participation of the immune system of the host. Four observations indicate that a competent immune system is essential for eradica-

tion of tumor cells by BCG. Adult mice (3) whose immune system was compromised by thymectomy and whole-body irradiation, and adult guinea pigs (2), intravenously infected with BCG, were unable to develop delayed sensitivity to purified protein derivative (PPD) of tubercle bacilli and to suppress tumor growth at sites of BCG infection. Guinea pigs (4) treated with antilymphocyte serum were unable to develop BCG-mediated regression of established intradermal tumors and to develop delayed sensitivity to PPD. Anergic patients with malignant melanoma (5) received an intralesional injection of BCG; intradermal metastases of the melanoma could not be eradicated by the BCG.

Impairment of immune reactions of the delayed-hypersensitivity type and, specifically, impairment of the ability to develop or express delayed sensitivity to PPD are correlated with the inability to kill tumor cells at sites of BCG infection. The corollary of this statement is that tumor killing by BCG requires an immune response by the host to BCG

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antigens. Tumor killing by BCG is abrogated by procedures that impair the development and expression of the cellular immune response of the host to mycobacterial antigens.

At this point, other ideas will be discussed regarding BCG's antitumor action. Increased activity of the reticuloendothelial system caused by BCG infection was correlated with antitumor effect (1). The precise relationship between increased activity of the reticuloendothelial system and suppression of tumor growth was undefined. Specific tumor immunity might develop more rapidly or to a greater degree after BCG infection. Adding some evidence to support this contention (6) our laboratory emphasized that one prerequisite for BCG's antitumor effect is the ability to develop and express a cellular immune response to mycobacterial antigens.

The location of BCG infection is critical in determining the outcome of therapy. Our laboratory has primarily studied the effects of BCG infection on tumors in the skin. BCG infection at the site of growth of the skin tumor is essential for tumor suppression. BCG infection adjacent to or contralateral to the tumor site does not inhibit tumor growth, nor does BCG infection before inoculation of tumor cells (2). In the guinea pig, BCG injected intradermally (intratumor injection) eradicates lymph node metastases (7). Acid-fast organisms are present in lymph nodes containing dying tumor cells (8).

Other investigators (1, 9-11) have shown that BCG administered systemically inhibits tumor growth in subcutaneous tissues, peritoneal cavity, lungs, and spleen. In these experiments, the site of tumor growth and BCG infection often coincided, *e.g.*, intraperitoneal infection followed by intraperitoneal inoculation of tumor cells and intravenous infection followed by intravenous inoculation of carcinogen or tumor cells. Successful systemic BCG therapy does not contradict the apparent requirement for BCG infection at the tumor site. Knowledge of distribution of BCG after infection by different routes, may improve therapy of internal tumors (table 1).

A wide variety of mycobacterial preparations are available to the clinician interested in cancer therapy. Preparations with potent antitumor activity share common characteristics which have been defined in experiments on animals and on man. In immunized and nonimmunized guinea

TABLE 1.—Distribution of BCG organisms

	iv*	id*
Liver	$6.16 \times 10^4$	$1.04 \times 10^4$
Spleen	$2.6 \times 10^4$	$2.6 \times 10^4$
Lungs	$9.8 \times 10^3$	$1.82 \times 10^3$
Lymph nodes†	$2.25 \times 10^4$	$1.1 \times 10^3$
Bone marrow	$2.88 \times 10^4$	0
Skin	—	0

\* C3H/HeN male mice were treated by intradermal (id) injection of  $3 \times 10^6$  BCG (5 mice) or by intravenous (iv) injection of  $3 \times 10^6$  BCG (5 mice); 30 days after injection of BCG, they were killed. In each group, organs were pooled and homogenized in phosphate-buffered saline. Log dilutions were plated on Dubos oleic agar. Numbers represent the average number of bacteria/organ 30 days after infection.

† Axillary and inguinal lymph nodes from iv group. Inguinal lymph nodes from id group.

pigs and mice, *living* BCG suppressed tumor growth (2, 3). In guinea pigs immunized to BCG, heat-killed BCG or mycobacterial extracts were partially effective. In nonimmunized mice, heat-killed BCG partially suppressed tumor growth (12). Living BCG is effective in immunized or nonimmunized hosts because it causes the development of an immune response of the delayed type to mycobacterial antigens. Killed BCG preparations fail in animals not immunized to mycobacterial antigens, for they do not immunize; *i.e.*, they do not provoke an immune response of the delayed type to mycobacterial antigens.

Recently, intralesional injection of PPD into tuberculin-sensitive patients caused regression of cutaneous lesions of mycoses fungoides and breast cancer (13). Mycobacterial preparations that elicit delayed-sensitivity reactions are effective in the tuberculin-sensitive individual.

Another characteristic of effective mycobacterial preparations is the development of a chronic inflammatory reaction at the tumor site. Tumor killing was studied in guinea pigs inoculated with a mixture of tumor cells and mycobacterial antigens. In a representative experiment, animals immunized to BCG and nonimmunized animals received injections containing tumor cells and PPD. Immunized animals developed delayed cutaneous hypersensitivity reaction to PPD; the reaction lasted 24-48 hours. Tumor was partially inhibited at sites of this type of reaction (14). On the other hand, injection of living BCG mixed with tumor cells led to an inflammatory reaction that persisted for about 3 weeks. Tumor was completely inhibited at sites of this reaction. We regard the ability of a mycobacterial preparation to elicit a chronic inflammatory



reaction in the skin of guinea pigs as the *sine qua non* of tumor suppression. The only nonliving mycobacterial preparation eliciting this type of chronic reaction also inhibited tumor.

Our laboratory recently became involved in studies to determine the components of mycobacteria responsible for tumor suppression. Experiments to date indicate that nonliving mycobacterial preparations can substitute for living mycobacteria as inhibitors of tumor growth in nonimmunized animals (15). The effective preparation contains BCG cell walls attached to droplets of mineral oil. The protoplasm of the mycobacteria is not essential for prevention of tuberculosis or tumor suppression. This cell-wall preparation is effective in treatment of established intradermal tumors in guinea pigs. The search for the molecules responsible for the biologic activity of mycobacteria may lead to the production of a standard, nonliving preparation highly active in inhibiting tumor growth. A molecule or a combination of molecules which elicits formation of granulomas is the chief subject of this search. Evidence suggests that, for optimal granuloma formation by mycobacteria, a lipid and protein molecule are required.

BCG-mediated tumor regression requires an adequate dose of BCG. The threshold dose of BCG that will initiate suppression of  $10^6$  tumor cells in the skin is about  $10^5$  living bacteria (2). A dose of  $10^6$ – $10^7$  living bacteria produces optimal tumor suppression. In therapy of internal tumors, consideration must be given to obtaining delivery of an adequate number of bacteria to the tumor target.

BCG-mediated tumor regression is limited by tumor size. Experiments on mice and guinea pigs indicate that  $>10^6$  tumor cells can be killed at sites of BCG infection. Intradermal tumors of 100 mg regress in  $>50\%$  of guinea pigs treated by intralesional injection of BCG; tumors of 500 mg regress in about 20% of guinea pigs treated by intralesional injection of BCG (7).

One idea of the mechanism of BCG-antitumor action relates to proposed augmentation of specific antitumor immune response. Dr. G. L. Bartlett performed critical experiments to determine whether specific tumor immunity was necessary for suppression of tumor growth at sites of BCG infection. Adult mice treated by thymectomy and whole-body X irradiation can express pre-existing cellular immunity but cannot develop cellular im-

munity to new antigens. Adult mice immunized to BCG and control, nonimmunized mice were thymectomized, X irradiated, and subsequently given injections of mixtures of living BCG and tumor cells. Immunized, immunosuppressed mice rejected the tumor at the BCG site but could not reject a subsequent challenge with tumor cells alone. Nonimmunized mice did not reject the tumor at the BCG site. BCG-mediated tumor cell killing may occur without the development of specific anti-tumor immunity (3). We have been unable to demonstrate in immunologically competent mice that BCG infection augments specific antitumor immunity.

Specific antitumor immunity may develop as a result of BCG-mediated tumor killing. One tumor (line 10) has been thoroughly studied in guinea pigs. This tumor is not antigenic by customary procedures for demonstrating tumor transplantation immunity. Specific tumor immunity develops when this tumor is suppressed at the site of BCG infection (16).

The understanding of the cellular and molecular basis for BCG-mediated tumor regression is gradually emerging from studies on the mechanism of cellular immunity to intracellular parasites (17, 18) and from studies on the components of the tubercle bacillus required for prevention of tuberculosis. Mycobacteria, after injection into the host, migrate to regional lymph nodes where they stimulate cells in the paracortical region of the node to become lymphoblasts. These lymphoblasts and their descendants leave the lymph node and enter the systemic circulation. Able to recognize antigens of the tubercle bacillus, these lymphocytes come in contact by chance with mycobacteria in the tissues. These specifically sensitized lymphocytes, interacting with mycobacterial antigens, then react by producing and secreting soluble molecules. These soluble molecules include factors that immobilize and activate macrophages. As a result of this process, macrophages are concentrated at sites in the tissues containing living BCG. The histologic equivalent of these concentrations of activated macrophages is the granuloma. These activated macrophages are the principal suspect for the cell responsible for tumor cell death at sites of BCG infection.

The evidence for the activated macrophage as the principal effector cell in BCG-mediated tumor killing is indirect. Histopathologic studies show that the characteristic feature of BCG-mediated

tumor killing is the presence of histocytes at sites of tumor cell death. Reactions characterized by the development of blast cells in the lymph node paracortex without histocytes do not lead to tumor cell death (8). Those characterized by the presence of macrophages containing phagocytized material without histocytes also do not lead to tumor cell death (19). Additional evidence includes: 1) Mice infected with intracellular parasites (toxoplasma, besnoitia) have increased resistance to tumor growth (20); macrophages from these infected animals possess potent tumoricidal growth *in vitro* (21); 2) macrophages obtained from the peritoneal cavity of normal guinea pigs inhibit tumor growth *in vivo* (22); 3) skin inflamed by the injection of migration-inhibitory factor resists tumor growth (23); and 4) skin inflamed by injection of turpentine or India ink also resists tumor growth (24).

A question not raised as yet in this review is whether BCG-mediated tumor regression displays tumor selectivity. Possibly the host during the cellular immune response to BCG in the tissues indiscriminately kills normal and tumor cells. Support for this idea, that normal cells are killed by the immune response to BCG, comes from the familiar appearance of the necrotizing ulcer developing after injection of living BCG into the skin and the well-known pulmonary changes in animals with tuberculosis. On the other hand, we have observed that agents, such as turpentine, that elicit a necrotizing reaction in the skin do *not* cause established tumors to regress. This important question of selectivity of the response has not been resolved.

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## Histopathology of *Mycobacterium bovis* (BCG)-Mediated Tumor Regression<sup>1, 2</sup>

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**SUMMARY**—A comparative histopathologic study was performed on inbred guinea pigs at the site of a transplanted syngeneic hepatocarcinoma and in the draining lymph nodes, in the presence and absence of *Mycobacterium bovis* strain bacillus Calmette-Guérin (BCG). BCG was injected into the growing intradermal tumor 7 days after transplantation when the tumor had metastasized to the first regional lymph node. The histopathology was compared with that of saline-inoculated tumors and with that of animals in which tumors had been surgically removed 7 days after transplantation. In this system guinea pigs died 60–90 days after intradermal injection of  $10^5$  hepatocarcinoma cells without BCG treatment. The results demonstrate that intradermal tumors completely regress after treatment with BCG and that regional lymph node metastases are eliminated. The mechanism is a BCG-mediated granulomatous reaction at both the tumor site and the regional lymph node. As detected histologically and ultrastructurally, histiocytes appear to be the major effector cells in this reaction. In this syngeneic tumor system, conventional lymphoproliferative response of the regional node, in the absence of histiocytosis, is insufficient to inhibit tumor growth. Additionally, treatment of these transplanted syngeneic tumors in guinea pigs with a single sensitization by vaccinia virus, oxazolone, or turpentine was compared with BCG therapy. Cellular reactions in the regional lymph nodes, which were characteristic of the development of delayed-type hypersensitivity, were not detrimental to the tumor and did not alter metastatic growth. The turpentine-induced inflammatory reaction at the tumor site was also ineffective in suppressing tumor growth. Both turpentine and oxazolone treatments, however, enhanced tumor growth in the skin.—*Natl Cancer Inst Monogr* 35: 345–357, 1972.

AN EXPERIMENTAL MODEL consisting of a transplantable syngeneic hepatocarcinoma in inbred guinea pigs has been developed to study the

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<sup>3</sup> National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

requirements of effective cancer immunotherapy (7). Studies with this model have demonstrated that nonimmunized animals challenged with living tumor cells before treatment can be cured by active immunization only if the number of tumor cells in the challenge inoculum is relatively small and the interval between challenge and treatment is short enough so that a palpable tumor is not present at the time of treatment (2).

In another approach, inbred male guinea pigs (Sewall-Wright strain 2) with palpable intradermal



tumors and metastases in the lymph node draining the tumor site can be permanently cured by the injection of living *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) into the skin tumor (3). In this experimental model, untreated guinea pigs die 60–90 days after intradermal injection of  $1 \times 10^6$  line-10 syngeneic hepatocarcinoma cells. Evidence has been presented indicating that nonlymphoid mononuclear cells are important in the process leading to tumor regression (4, 5). This report deals with a study of the histopathology of the site of the regressing tumor and the draining lymph node.

## HISTOPATHOLOGY OF TUMOR REGRESSION AFTER BCG INJECTION

Of paramount importance in understanding the mechanism of BCG-mediated tumor and metastasis regression is the histopathology of the events of the tuberculin reaction in the tumor site and the draining lymph node. These results will be of more than just academic interest, since as described recently by Spector (6), the histology of the host response to tubercle bacilli is still the subject of debate. The cutaneous reaction to living BCG appears essentially to be a biphasic inflammatory response, consisting of an immediate (polymorphonuclear) reaction followed by a vigorous mononuclear response. The hematogenous origin of the mononuclear cell in the reaction site can be assumed, based on studies by Kosunen *et al.* (7) and Volkman and Gowans (8) in normal animals. These cells have been generally classified as macrophages of monocytic origin, derived from the bone marrow (9).

We evaluated the histopathology at the skin site (upper right quadrant) of a transplanted syngeneic hepatocarcinoma and in the draining lymph node in the presence and absence of *Mycobacterium bovis* strain BCG (Phipps strain TMC 1029). Twenty-four  $\times 10^6$  living BCG were injected into the intradermal tumor 7 days after transplantation, a time suspected to be the earliest stage of metastasis to the draining lymph node. The histopathology was compared to that of saline-inoculated tumors and to that of animals in which tumors had been excised at day 7. Normal guinea pigs receiving a single intradermal BCG injection were also evaluated as controls (10).

It is important to qualify the terminology to be associated with these descriptions, because of the

difficulty of interpreting the extensive literature on classic tuberculin granulomatous disease. The general term histiocyte is used to refer to a cell type distinguishable from active macrophages or phagocytic mononuclear cells and consistent with the characteristics of the epithelioid cells associated with granulomatous reactions (11). It is accepted that these may be different forms of the same cell type (12). Also, it is recognized that in the early host response, phagocytosis of a limited number of bacilli by macrophages initially activates this cell compartment, as described by Dannenberg (13). However, the general absence of active phagocytosis and representative numbers of cell inclusion bodies in the enlarged, activated compartment of responding cells tends to argue against phagocytosis as their sole primary function. We reject the terms “epithelioid” and “epithelial-like cell” because they are based on a superficial resemblance and are misleading with respect to the origin and function of the cell.

## Tumor Site and Lymph Node Before Treatment

Histologic examination 4 days after tumor cell injection showed tumor cells to be confined to the skin. They were retained within the reticular layer of the dermis, with subcutaneous extension.

The chain of lymph nodes draining the tumor site were carefully selected and examined histologically and ultrastructurally. The first ipsilateral node was the superficial distal axillary (SDA) lymph node. Anterior to this was the proximal axillary (PA) lymph node. Similar dissection was performed on the contralateral side. Both the SDA and PA lymph nodes, the first and second nodes draining this tumor site, were free of tumor cells and appeared normal at 4 days. These nodes possessed intact nodular cortical (thymus-independent) and paracortical (thymus-dependent) regions and occasional germinal centers in the nodules of the cortical zone. The marginal sinus surrounded the cortex, and we considered that the population of cells of the marginal sinus represented cells from the afferent lymph. A normal medulla consisted of both cords and sinuses (fig. 1).

By day 7 the tumor had grown mainly within the subcutaneous regions of the skin, and a slight mononuclear cell infiltration consisting of lymphocytes and occasional histiocytes surrounded the penetrating edge of the tumor mass. Tumor cells could

be observed in the SDA lymph node at 7 days. The tumor cells, both intact and in mitosis, were confined to the subcapsular marginal sinus (fig. 2). Thus it was established that metastasis had occurred in the first draining node before treatment or excision of the tumor. No evidence of metastasis could be observed in any other lymph nodes of either the ipsilateral or contralateral side.

### Comparison of Saline or BCG Intralesional Injection and Tumor Excision

One day after saline injection, mononuclear cells infiltrated the subcutaneous border of the tumor. This infiltration consisted mainly of lymphocytes and occasional histiocytes. At day 4 after saline injection (day 11 after tumor cell injection) the tumor mass had visibly increased in size without quantitative or qualitative change of mononuclear cell infiltration. At 25 days after saline injection there was a massive tumor growth (fig. 3) and a marked increase in the associated connective tissue component of the skin. Whether this is a characteristic of the tumor mass or a response of the local connective tissue to the invading tumor is undecided. Also, within the tumor mass there was a deep infiltration of lymphocytes and occasional histiocytes.

In contrast to the tumor site alterations after

saline injection, 24 hours after BCG injection an acute inflammatory reaction was observed in the tumor site. This was characterized by edema and both perivascular and intervascular infiltration of polymorphonuclear and mononuclear cells. Grossly, the underside of the tumor was hemorrhagic and distinct from saline-inoculated tumors at this interval. Eight days after BCG injection, tumor cell nests were surrounded by fibrotic components of the dermis, and many tumor cells showed signs of degeneration. The tumor site regressed by day 15 and was generally fibrotic with some evidence of syncytial histiocytosis. No intact tumor cells could be distinguished, while there were numerous areas of focal necrosis.

The major histologic changes in the draining SDA lymph node after tumor excision and after saline or BCG intralesional injections are compared in table 1. Except for a progressive metastatic growth of tumor cells, no other marked alterations occurred in SDA lymph nodes after saline injection. At 25 days after saline injection, a greatly enlarged SDA lymph node was entirely metastatic, with some small portions of lymphoid components (fig. 4). The PA and contralateral lymph nodes, while they showed no metastases, did contain a degree of reticuloendothelial activity, best described as histiocytosis.

TABLE 1.—Major histologic changes in the draining lymph node following various treatments of intradermal tumor

Time after treatment (days)	Major histologic change	Treatment					
		Tumor excision	Saline	BCG	Vaccinia	Oxazolone	Turpentine
1	Tumor cells in marginal sinus	+	+	+	+	+	+
4	Tumor cells in marginal and medullary sinuses	+	+	+	+	+	+
	Granulomas in cortex and paracortex	—	—	+	—	—	—
	Histiocytes and lymphocytes in medullary sinuses	—	—	+	—	—	—
	Active macrophages and lymphocytes in medullary sinuses	—	—	—	+	+	+
	Extensive proliferation of blast cells in cortical and paracortical regions	+	—	—	+	+	—
8	Tumor cells throughout node	+	+	+	+	+	+
	Coalesced granulomas	—	—	+	—	—	—
	Germinal centers	—	—	—	+	+	—
	Plasma cells	—	—	—	+	+	—
	Extensive proliferation of blast cells in cortical and paracortical regions	+	—	—	+	+	—
25	Metastatic lymph node	+	+	—	+	+	+
	Dilated, cystic sinuses	+	+	—	+	+	+
	Syncytial histiocytosis	—	—	+	—	—	—
	Extensive proliferation of blast cells in cortical and paracortical regions	+	—	—	—	—	—

A unique feature of the SDA lymph nodes in the tumor-excision group was a marked hyperplastic germinal center reaction in the cortical area (fig. 5). This was a very prominent alteration of the cortex, implying a unique reaction to metastatic tumor cells not seen in the presence of a growing tumor, as in the saline-inoculated group.

The metastases in the subcapsular marginal sinus were more extensive 1 day after BCG, compared to the SDA nodes of saline-inoculated or tumor-excision guinea pigs (fig. 6). We assume that there was a greater influx of tumor cells or tumor emboli via the afferent lymphatics as a result of the acute inflammatory response and the vascular alterations at the tumor site. In acid-fast stained preparations, mycobacteria were detected in the marginal sinus; the organisms were associated with macrophages seen in the marginal sinuses and were never seen in direct contact with tumor cells. On the ultrastructural level, encapsulated organisms were detected intracellularly in occasional reticuloendothelial cells (fig. 7a).

At day 4 after BCG injection the architecture of the ipsilateral SDA lymph node was markedly altered (fig. 8). Tumor cell penetration into the cortex had destroyed the follicular configuration, and an absence of active germinal centers was noted. Noncaseous granulomas were observed throughout the cortex, bordering the metastatic rim of the node. Small numbers of mycobacteria were occasionally detected in these granulomas. Histologically the focal granulomas consisted of concentrations of histiocytes, and mitotic figures were common in these structures. The characteristic features of the histiocytes involved in granuloma formation are illustrated in figure 7b. Each cell has a large nucleus with the heterochromatin dispersed at its periphery in one or more discrete nuclei. The principal organelles of the voluminous cytoplasm are numerous small cisternae of rough-surfaced endoplasmic reticulum and large, electron-lucent mitochondria. The intercellular space is filled with the interdigitation of the slender, finger-like processes of the cytoplasm of these cells. In the subcapsular and medullary sinuses of the regional draining lymph nodes these histiocytes and lymphocytes induced degeneration, although not phagocytosis, of metastatic tumor cells. In the medullary sinuses of these nodes the histiocytes were frequently in close association with tumor cells, and some of the tumor cells showed definite signs of degeneration (fig. 7c, d; fig. 9).

Eight days after BCG injection (15 days after tumor cell injection) the architecture of the ipsilateral SDA lymph nodes consisted of coalesced granulomas occupying the major portion of the cortical and paracortical regions of the node. In some areas the granulomatous reaction had become generally infiltrated with polymorphonuclear cells. Residual tumor cells within these reaction centers showed signs of degeneration (fig. 10). There was a marked decrease in the number of tumor cells in these nodes. Granulomas but no tumor cells were observed in the ipsilateral axillary nodes.

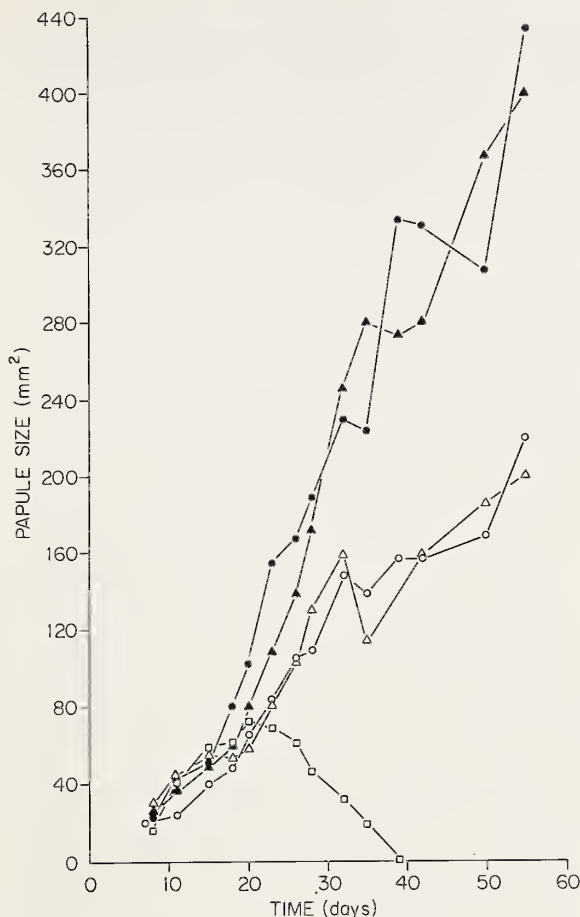
At 25 days after intrasiesional BCG injection the ipsilateral SDA nodes were reduced in size from the previous interval (fig. 11), and a syncytial histiocytosis occupied most of the node (fig. 12). Distinct sinuses were rare, and areas of focal necrosis were seen throughout the node. Small but discrete regions of lymphoid components could still be observed.

### Comparison of BCG With Inflammatory and Sensitizing Agents

We have also compared BCG-induced reactivity and the reactions induced by turpentine, vaccinia virus, and oxazolone in the interdermal tumor and the regional lymph node (14). Text-figure 1 shows the effects of various treatments on the rate of tumor growth. Between 8 and 20 days all treatments slightly increased papule size, compared to the saline-inoculated controls, as a result of the immediate inflammatory response at the site of sensitization. Twelve to 13 days after intratumor injection of BCG, the tumors began to regress and eventually disappeared. After the initial inflammatory response, the vaccinia virus-treated tumors grew at the same rate as the saline-injected controls. Both turpentine- and oxazolone-treated tumors grew faster than tumors of saline-inoculated controls. At day 55 the tumor mass in oxazolone- and turpentine-treated guinea pigs was about twice that of the control animals.

Table 1 summarizes the major changes in the regional lymph nodes during the first 25 days after treatment from these various agents. In the regional lymph nodes responding to intratumor injections of vaccinia virus and oxazolone, in contrast to the granulomatous reactivity of BCG-treated animals, there was extensive proliferation of cells in the thymus-dependent region (paracortex) of the node (fig. 13). Macrophages laden with pig-





TEXT-FIGURE 1.—Tumor growth calculated from mean papule size ( $\bar{x}^2$ ) after various treatments: O, saline (controls); □, BCG; △, vaccinia virus; ●, oxazolone; ▲, turpentine.

ment were abundant in the medullary sinuses of these nodes, as well as in the nodes of turpentine-treated animals. The reaction observed in the lymph nodes of oxazolone- and vaccinia virus-treated animals was similar to that which accompanies the development of delayed-type hypersensitivity.

At 8 days after treatment with oxazolone and vaccinia virus, the hyperreactivity of the paracortex of the node was still present, with active germinal centers in the cortex and plasma cells in the medullary sinuses. No such reactivity was observed in the nodes draining turpentine-inoculated tumor sites.

At 25 days after treatment, the draining regional lymph nodes in the turpentine-, oxazolone-, and vaccinia virus-treated guinea pigs were metastatic, possessing dilated fluid-filled spaces. In the nodes

draining oxazolone- and vaccinia virus-treated tumors, occasional active germinal centers could still be observed, with large areas of polymorphonuclear cell abscesses. The reaction of the regional lymph nodes at 25 days in the oxazolone- and vaccinia virus-treated animals was in marked contrast to the tumor-free regional nodes of BCG-treated tumors.

## DISCUSSION

There are three major components of the immune system: humoral antibody, cytotoxic lymphocytes, and reticuloendothelial cells such as mononuclear cells, histiocytes, and macrophages. As pointed out by Gorer (15), the effectiveness of these components is not equal at all sites and their individual or combined efficiency may be limited by anatomical factors. In a discussion of the limitations of these three components of the immune system in host-tumor interactions, Alexander (16) pointed out that the "success of antigenic tumor occurs for the same reasons that permit infectious diseases to establish themselves and to persist in immunologically competent hosts." That is, survival of tubercle bacilli in the heart valve is likened to the success of an antigenic tumor. Both occur because anatomic factors limit the full expression of the effector arms of the immune system. Accepting this, there is increasing evidence of a further limitation of the immune system in the host-tumor interaction, which is that two effector components of the immune system interact competitively or antagonistically. Antibody specific to tumor transplantation antigen has been shown *in vitro* to prevent sensitized lymphocytes from being cytotoxic to tumor cells (17); and it has been suggested that tumor antigen-antibody complexes may neutralize the activity of cytotoxic lymphocytes by binding to surface receptor sites (18).

In general, less attention has been devoted to the reticuloendothelial component and its activity in host-tumor interaction, probably because it does not demonstrate the degree of immunologic specificity expressed by antibody or sensitized lymphocytes. The major limitation of this effector arm of the immune system is cell concentration at the primary or disseminated tumor sites. Alexander (26) demonstrated that peritoneal "macrophages" isolated from animals immunized against a specific tumor or from animals with progressively growing tumors can destroy tumor cells *in vitro*. Phago-

cytosis was not involved in this cytotoxic reaction. The paradox here is that the tumor grows progressively in an animal that has sufficient number of peritoneal mononuclear cells to destroy it. Zbar *et al.* (19) elucidated this point in studies that demonstrated the failure of a transplanted guinea pig hepatocarcinoma to survive at a skin site in which erythema and induration had been induced by prior injection of a "macrophage-rich fraction" of peritoneal cells. Thus a critical concentration of peritoneal cells was capable of killing the tumor by virtue of a nonspecific response.

The effective antitumor reaction in BCG-treated animals is a nonspecific granulomatous reaction, characterized by an intense proliferation of stromal reticuloendothelial components and histiocytes. In contrast, lymphoproliferative cellular reactions characteristic of developing delayed-type hypersensitivity and nonspecific inflammatory reaction do not have detrimental effects on the tumor. It is apparent from the comparison of BCG-treated normal and BCG-treated tumor-bearing guinea pigs that the qualitative and quantitative characteristics of the granulomatous reaction, both at the tumor site and in draining lymph nodes, is only a function of a specific immunologic reaction provoked by the microorganism. Granulomatous inflammation is generally defined as the reticuloendothelial cell response to tissue injury, mediated by a poorly soluble substance (11). It may be characterized as a foreign-body response as to a colloidal substance, generally involving a simple phagocytic process, or as a more intense proliferative reaction, involving primarily the stromal reticuloendothelial components and/or histiocytes. The granulomatous reaction is characteristic of the BCG-tumor system in the present study.

The question of immunologic specificity, or of developed immunity in the BCG granulomatous reaction in a syngeneic tumor system, is difficult to evaluate at present. It has been established that the BCG-mediated regression of transplantable syngeneic hepatocarcinoma and elimination of regional lymph node metastases is a two-step process (5). The first step is immunologically specific for BCG antigen. We have further data to support this analysis, based on the inability of BCG to induce tumor regression in animals made immunologically incompetent by antilymphocyte therapy (unpublished data). The second step, which is effective in destroying tumor cells, is probably immunologi-

cally nonspecific. There is evidence, however, that during tumor regression specific tumor immunity is developed, mediated by lymphocytes as well as detectable antitumor-specific antibody. It has been demonstrated that the syngeneic hepatocarcinomas used in the present study are rejected when they are reintroduced into guinea pigs that have recovered from tumor cell-BCG granulomatous reaction (20). These results suggest that lymphocyte sensitization does occur during the granulomatous reaction, and based on studies by Olson *et al.* (21), which demonstrate that regional lymph nodes draining BCG sensitization sites are anergic, one would have to assume that the developing lymphocyte sensitization during a BCG granulomatous response probably occurs systemically in peripheral lymphatic tissue as well as in the draining lymph nodes.

It is important that we consider the mechanism of BCG-induced tumor regression and elimination of regional lymph node metastases as a nonspecific reaction mediated by the physiologically altered and possibly detrimental environment created by the granulomatous reaction at the tumor site in the draining lymph node. Constriction of postcapillary venules has been described in lymph nodes reacting to certain delayed hypersensitivity antigens (21, 22). There is good reason to believe that this condition is also created by BCG in the node, causing infarction in the affected areas, and that as a result tumor cells which normally grow well in draining lymph nodes (as seen in the saline-inoculated and tumor-excision animals) find the environment of a granulomatous reaction detrimental to growth. While this may account for part of the destruction of the metastatic growth, it still does not completely explain the subtle histiocyte-tumor cell interactions in the lymph node sinuses, where there are obvious signs of tumor cell degeneration. This response of histiocytes and tumor cells is reminiscent of the "activated" histiocyte-tumor cell interaction described by others in *in vitro* as well as *in vivo* systems (23-26). The cytopathic mechanism by which such "activated" histiocytes destroy tumor cells is not completely understood; however, it does not appear to be simply a phagocytic process. Furthermore, there is at present little or no evidence to support the contention that an immunologically specific histiocyte hypersensitivity to the tumor is developed. Also, the effectiveness of the granulomatous reaction in completely eliminating tumor and metastases is most certainly based on the propensity

of this tumor system to metastasize to the lymph nodes via the afferent lymphatic, as is the case for many tumors in man, rather than to other sites via the bloodstream. This is a critical aspect of the system, since the early inflammatory reaction enhances general dissemination of tumor cells, a consequence of vascular alterations of the primary transplantation site.

It might be reasonable at this time to consider that a natural example of such a beneficial host-tumor interaction is the prognostically favorable reaction of draining lymph nodes to a prolonged exposure to neoplasia, such as carcinoma of the breast and stomach in man, originally described as "syncytial" and "sinus histiocytosis" by Black and Speer (27). At present we are prone to make a histopathologic correlation between the general granulomatous reaction induced by BCG and syncytial histiocytosis in man.

In the light of the demonstrated success of intralesional injection of BCG in causing regression of transplanted syngeneic tumors and eliminating regional lymph node metastases, a stronger consideration should be made of the use of induced granulomatous reactivity as primary treatment as well as an adjunct of any immunotherapeutic or chemotherapeutic model for the treatment of tumors. It is possible that tumor growth normally precedes the development of specific cell-mediated immunity to a degree which constantly favors the tumor system. The induction of a granulomatous reaction at the tumor site and in the regional node during the early stages of metastasis would confine and destroy the major mass of tumor, at the same time releasing processed tumor antigen. Thereby, the major portion of the tumor would be destroyed presumably with release of tumor antigen as a result of concentration of histiocytes, allowing for the development of a systemic cell-mediated immunity capable of reaching and eliminating any tumor cells that by-passed or escaped the granulomatous reaction.

The major difference between tumor excision and the induced granulomatous reaction is the quantitative difference in the systemic release of processed tumor antigen, and thus in the degree of specific cell-mediated immunity. Furthermore, an organism such as BCG can distribute its activation capability both at the tumor site and in the regional and perhaps other peripheral nodes, the major location of tumor cells during the early intervals of the

response. That this is important is seen in the fact that when BCG is injected into tumors later than 7 days after transplantation, when the tumor has increased in size and metastasis has become more widespread and disseminated, it is considerably less effective (20). The experiments described and the explanation given here should provide a basis for further experimental studies of the advantages of this tumor-killing mechanism in conjunction with development of specific tumor immunity.

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FIGURE 1.—Proximal lymph node SDA draining tumor site 4 days after tumor cell injection. MS = marginal sinus; GC = germinal center; C = cortex; PC = paracortex; S = medullary sinuses; MC = medullary cords. Hematoxylin and eosin.  $\times 30$

FIGURE 2.—Tumor cell metastasis in subcapsular marginal sinus (MS) of SDA lymph node 7 days after transplantation. Hematoxylin and eosin.  $\times 300$

FIGURE 3.—Bisected tumor in the SDA lymph node (*upper right*) and contralateral lymph node (*upper left*) 25 days after saline injection.

FIGURE 4.—Metastatic SDA lymph node after intratumor saline injection. Note residual lymphatic tissue (*arrows*) and cystic condition of node. Hematoxylin and eosin.  $\times 30$

FIGURE 5.—SDA lymph node 25 days after tumor excision. Note hyperplastic germinal centers (GC) and extensive metastasis (M). Hematoxylin and eosin.  $\times 125$

FIGURE 6.—Tumor cells intermixed with histiocytes and macrophages (*arrows*) in the marginal sinus of the SDA lymph node of a BCG-inoculated tumor-bearing guinea pig 1 day after BCG (8 days after tumor transplantation). Hematoxylin and eosin.  $\times 300$



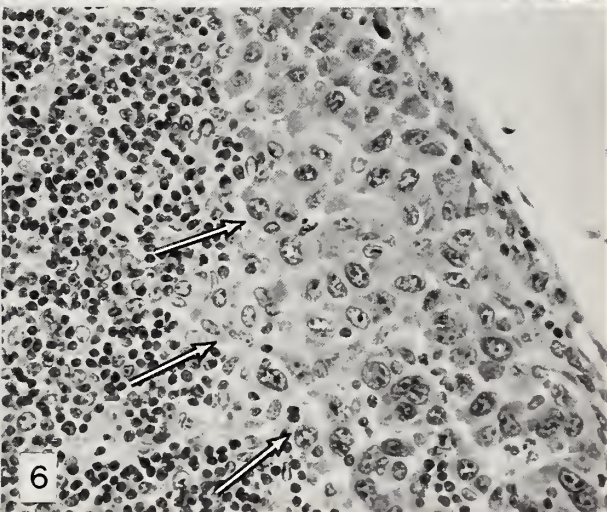
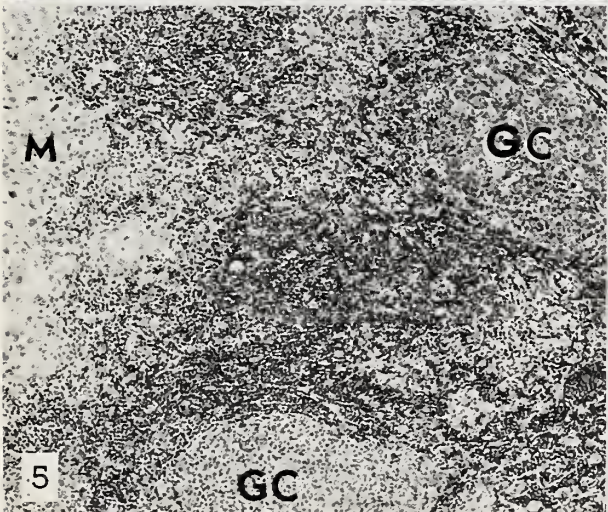
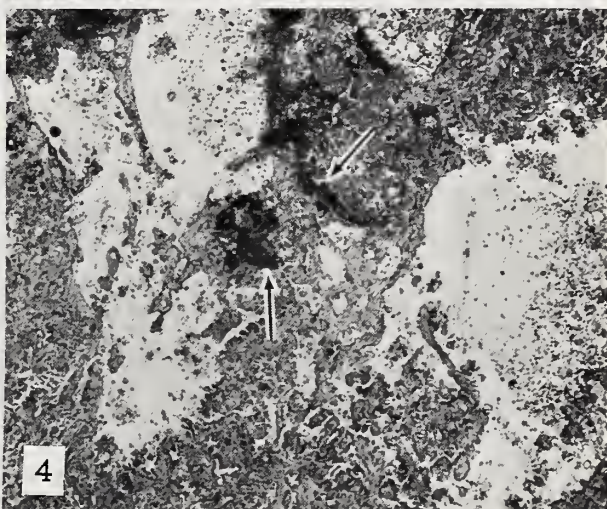
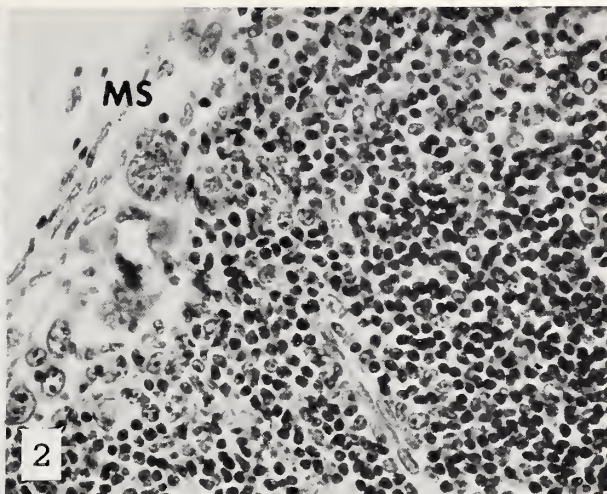
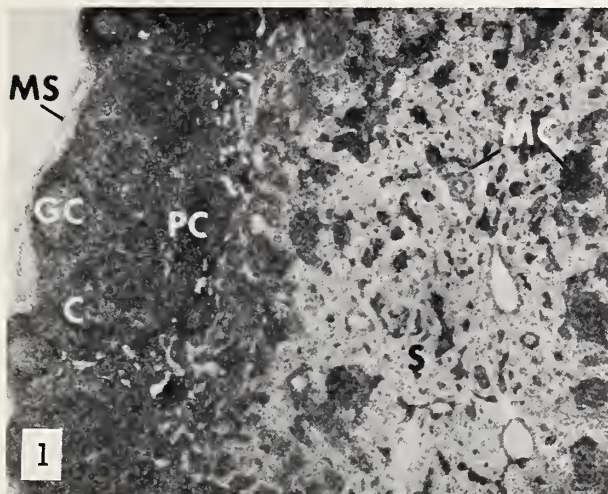


FIGURE 7.—Anatomy of stimulation of a cell-mediated anti-tumor response. *a*) BCG organism, cut in cross section, within a phagosome of a histiocyte in lymph node draining tumor site.  $\times 41,000$  *b*) Interdigitating histiocytes forming a “syncytial” granuloma in draining lymph node after stimulation with BCG.  $\times 5,700$  *c*) Three histiocytes and a lymphocyte in contact with a degenerating metastatic tumor cell in subcapsular sinus of lymph node draining tumor site 8 days after stimulation with BCG.  $\times 5,500$  *d*) Configuration suggestive of fusion of plasmalemmae of histiocyte (H) and tumor cell (T).  $\times 24,000$



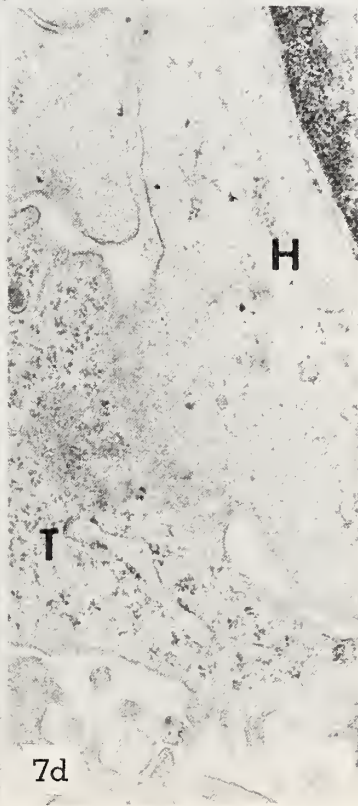
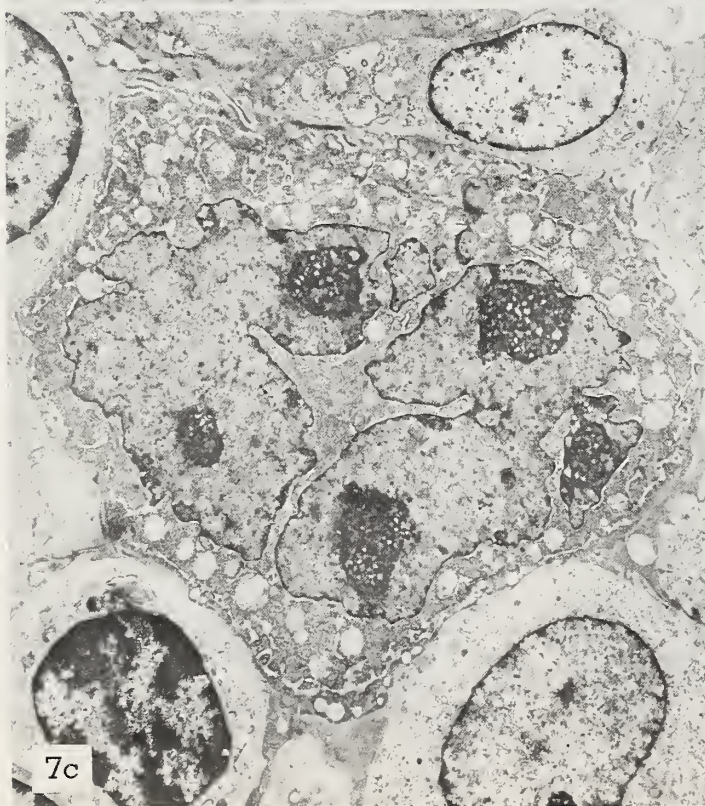
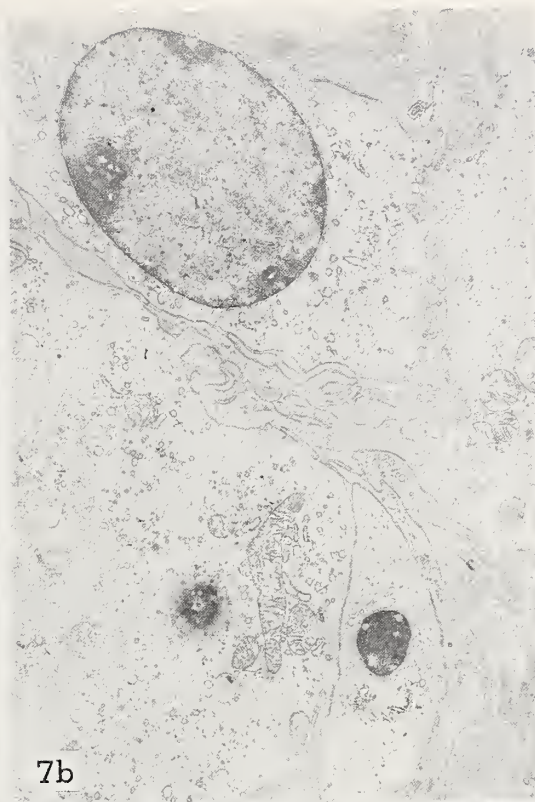
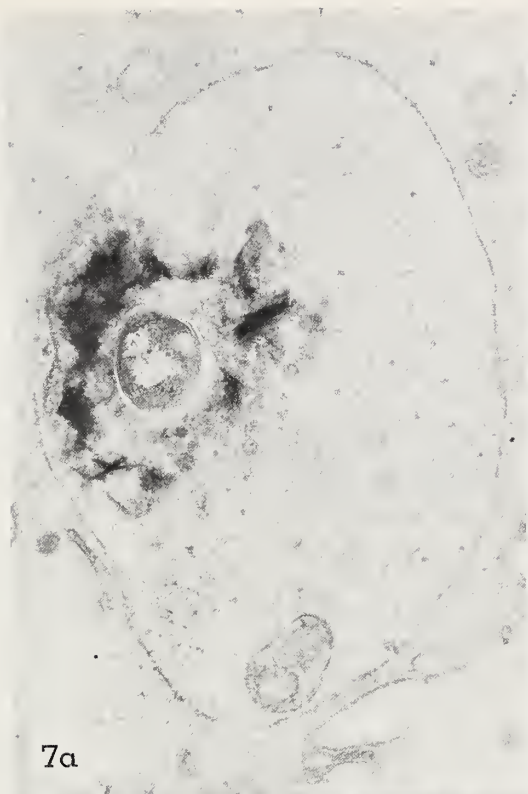


FIGURE 8.—SDA lymph node draining BCG-inoculated tumor 4 days after BCG injection. *Note* extensive metastasis (*upper right*), histiocyte-infiltrated medullary sinus (*lower left*), and granulomas (*arrows*). Hematoxylin and eosin.  $\times 30$

FIGURE 9.—Degenerating tumor cells (*arrow*) in close association with sinusoid histiocytes. SDA lymph node 4 days after intratumor BCG injection. Hematoxylin and eosin.  $\times 300$

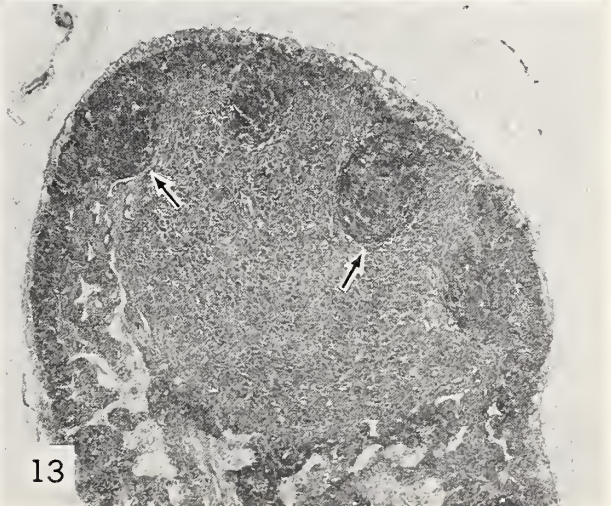
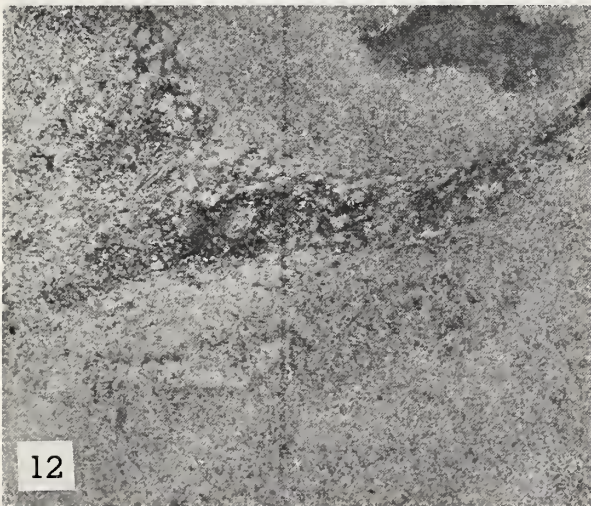
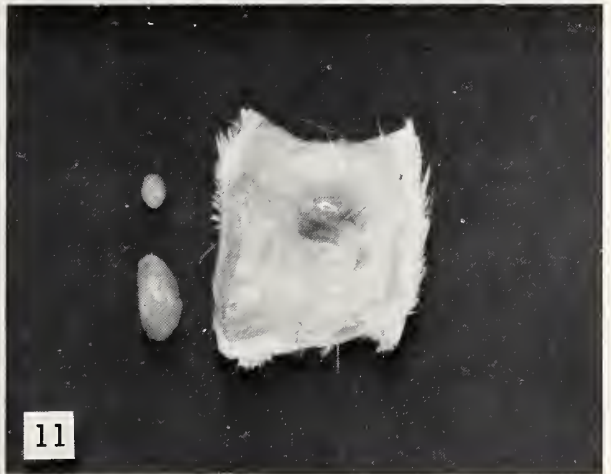
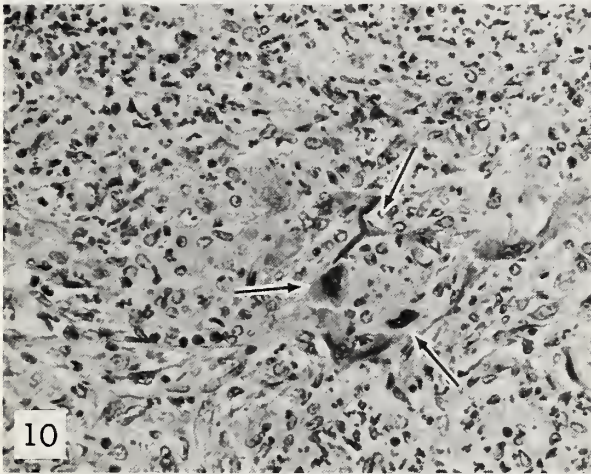
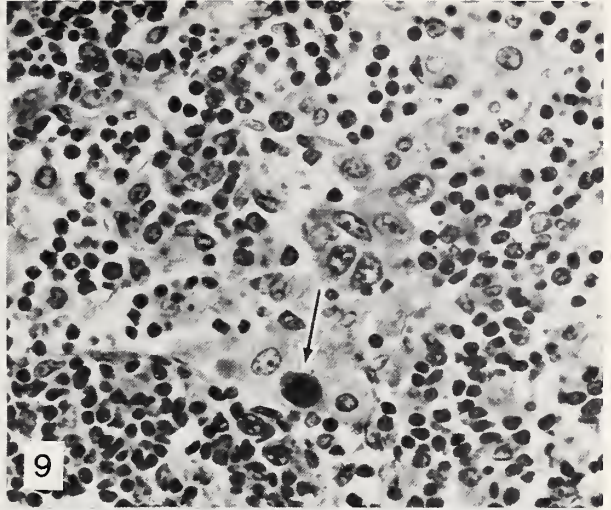
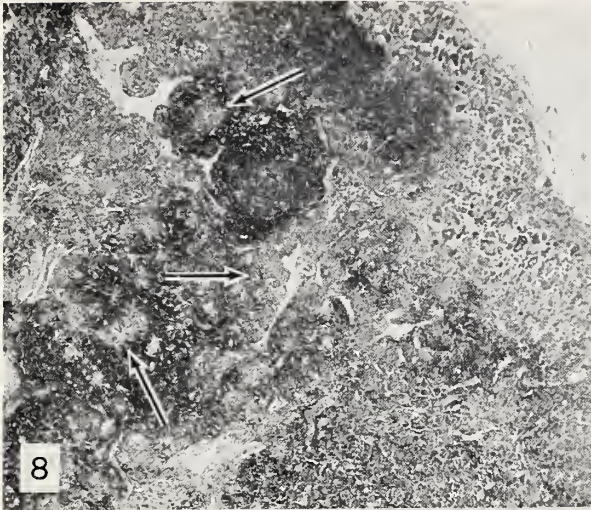
FIGURE 10.—Degenerating tumor cells (*arrows*) trapped in granulomatous reaction centers of SDA lymph nodes 8 days after intratumor BCG injection. Hematoxylin and eosin.  $\times 300$

FIGURE 11.—Remnant of tumor site 25 days after BCG injection. SDA lymph node (*bottom left*) and contralateral lymph node (*top left*).

FIGURE 12.—Syncytial histiocytosis of SDA lymph node 25 days after intratumor BCG injection. *Note* residual lymphoid component (*center*) and focal necrotic mass (*upper right*). Hematoxylin and eosin.  $\times 30$

FIGURE 13.—SDA lymph node draining tumor site 4 days after external application of oxazolone. *Note* distinct follicles in cortex (*arrows*) and the distended paracortex. Hematoxylin and eosin.  $\times 30$









## DISCUSSION

**E. Klein:** I would like to point out that this classical tumor model, which was devised by Dr. Rapp and which is the first one to be useful for immunotherapeutic monitoring, certainly has been most enlightening for clinical studies. Those who are interested in clinical investigation of tumor immunotherapy ought to be concerned about the precise system that the preceding authors have presented. It is the first system (a Model-T system), and others are being developed. We ought to bear in mind that at comparable phases in other areas of cancer therapy, such as radiation or chemotherapy, we did not have a system that allowed this kind of rational approach. The floor is open.

**G. Mathé:** It is interesting to see, as Dr. Hanna showed us, that histiocytes seem to play a major role and also, as Dr. Black showed us, that histiocytosis reveals a good prognosis in breast carcinoma. Maybe the immunologist must focus on histiocyte-macrophage studies instead of lymphocyte studies.

Dr. Zbar, I have 2 questions: With regard to humoral immunity, you said that if you inject bacille Calmette-Guérin (BCG) into a site other than the tumor you get nothing. Have you studied the results of decreasing the number of tumor cells or of injecting BCG and cells at a site other than the tumor?

**B. Zbar:** We have not decreased the number of tumor cells inoculated and studied the effects of the systemic BCG infection. In terms of making a vaccine with BCG and tumor cells, we have done this type of experiment. But this vaccine is ineffective against an established tumor. It is effective, however, in an animal with a very minimal tumor burden, of the order of  $\leq 10^6$  cells.

**J. H. Wallace:** Dr. Zbar, have you attempted to see what effect prior sensitization to mycobacteria would have on BCG injection, *i.e.*, in animals who were sensitive already to mycobacteria? Is tumor production accelerated?

**Zbar:** I did not make this point. When one uses the living bacteria, or the cell wall attached to oil droplets, it is not essential that the animal be previously immunized to BCG. We see no difference in therapeutic effect, depending on whether the animal was immunized or not. If the animal is immunized, there does not seem to be any additional benefit accruing to him.

**Klein:** Is there any detriment, Dr. Zbar?

**Zbar:** Both Dr. Bartlett and I have seen cases of what might be described as "death within 24 hours after inoculation of the material" in an animal who had been previously immunized with BCG. This has been a very infrequent observation. However, we have never seen anything like this when one starts with an animal who is not immunized to the bacteria.

**Wallace:** You stated as one reason for the response to this bacterial material the fact that, when you gave large doses of BCG intravenously, you apparently got no abrogation of the tumor. Do you really have evidence that these animals were unresponsive to mycobacterial protein?

**Zbar:** Yes. I didn't stress this, but on every occasion in which we used any form of immune-depressing mechanism, these animals were tested either with purified protein de-

rivative (PPD) of the tubercle bacillus or the heat-killed bacteria. In the case of intravenous injection of BCG, these animals were unresponsive to antigens of the tubercle bacillus. This observation was made, incidentally, about 20 years ago. This is not our observation.

**R. W. Baldwin:** I want to reinforce what Dr. Zbar said about the treatment of tumors at different sites. As Dr. Zbar knows, we are studying the same sort of effect in rat hepatoma and sarcoma systems, following Dr. Rapp's introduction of this system, and we now know that we can completely eliminate metastases within the lungs even when BCG is given as a single dose up to 7 or 10 days after the implantation of tumor cells at that site.

**G. B. Mackaness:** In your model, Dr. Zbar, it is very clear that the rejection of an established tumor with BCG requires the injection of BCG into that tumor. But have you looked at the fate of metastases when the BCG is injected adjacent to the primary tumor mass and thus has an access to the lymph node in which a metastasis is located? I would suspect that, if followed for any length of time, the metastasis could be inhibited although the primary tumor may continue to grow.

**Zbar:** This is an intriguing question, Dr. Mackaness. We have done experiments along this line which on the surface do not make sense to me. We excised the tumors when we knew there would be metastatic cells in the lymph node, and we injected the bacteria just adjacent to the site of excision.

We assumed that the bacteria would drain into that lymph node, which we could recognize by its enlargement. This did happen, but there was no therapeutic effect by this experimental strategy. I do not understand this experimental result. I would have expected that this inoculation of bacteria reaching draining node, combined with excision of the tumor, would have produced a beneficial effect, but we saw no such result.

**D. W. Weiss:** Whereas I fully accept the very elegant findings that Dr. Zbar has reported, it would be pertinent to note that, with a variety of other mycobacterial agents or their fractions, it is not necessary to inject the material directly into or near the tumor focus in order to obtain marked protective therapeutic effects.

The same effect, or, in our hands, an even better effect, can be obtained when these materials, such as the methanol-extraction residue (MER) fraction, are injected into a site entirely unrelated to the tumor site.

It might be worth while to add this to the discussion so as not to leave one with the impression that, in a general way, mycobacterial entities must be in direct proximity to the tumor focus.

**S. Deodhar:** Dr. Zbar, did you mention that you injected BCG intravenously? Were you trying to study the effect of producing specific tolerance to BCG on the tumor or its progression rather than using a method such as anti-lymphocyte globulin which produces a generalized immunosuppression? Have you studied the effect of producing tolerance to BCG on tumor progression?

**Zbar:** All we wanted to do was to impede or retard the

ability of the host to respond to antigens of the tubercle bacillus. I'm not sure what effects we produced. We produced the desired experimental results. I'm not sure whether that was tolerance or some other phenomenon.

**Klein:** Dr. Hanna would like to answer Dr. Weiss's question.

**M. G. Hanna:** One has to keep in mind that there is a big difference between the effects of BCG in mice and rats and the effects of BCG in guinea pigs. I failed to mention that, when one injects live BCG into mice or rats, the viable organisms can be detected months later. While in the guinea pig, the BCG organisms cannot be detected after day 4, which corresponds to the results that have been previously obtained at the National Cancer Institute. Dr. Zbar found

that the response can be abrogated by injecting live BCG and creating a systemic infection. It's simply a matter of not allowing the concentration of histiocytes at the tumor site to rise to a level which is essential both for the reaction and for killing tumor cells.

**T. Mariani:** Dr. Hanna, we have seen the same exquisite histology of the germinal centers you have shown as we have seen with the passage of a lymphoma 12 hours after tumor transfer. Have you recorded that exquisite intimacy of that histiocyte to the tumor cell with time-lapse photography?

**Hanna:** We have a film which is being developed.

**Klein:** Dr. Mathé.



## Attempts at Immunotherapy of 100 Patients With Acute Lymphoid Leukemia: Some Factors Influencing Results<sup>1</sup>

G. Mathé, P. Pouillart, L. Schwarzenberg, J. L. Amiel, M. Schneider, M. Hayat, F. De Vassal, C. Jasmin, C. Rosenfeld, R. Weiner,<sup>2</sup> and H. Rappaport,<sup>3</sup> *Unité de Développement Thérapeutique, Institut de Cancérologie et d'Immunogénétique, Hôpital Paul-Brousse<sup>4</sup> and Service d'Hématologie de l'Institut Gustave-Roussy,<sup>5</sup> 94-Villejuif, France*

**SUMMARY**—Our experimental models of active immunotherapy of leukemia employed immune stimulation after the establishment of disease in subcutaneously grafted L1210 leukemia, intravenously grafted Rauscher and E $\phi$  K1 leukemia, and spontaneous AKR leukemia. We demonstrated that active immunotherapy induced tumor regression and cured some animals. We showed that active immunotherapy was effective if the tumor cell load was  $\leq 10^5$  cells at the onset of active immunotherapy. This effective threshold could be achieved either by grafting  $10^5$  cells initially or by reducing a higher number of grafted cells by chemotherapy. We applied these criteria derived from animal models to man to treat acute lymphoid leukemia (ALL). The tumor cell load was reduced to the point of apparently complete remission by chemotherapy and further lessened by cell-reducing complementary systemic chemotherapy combined with chemoradiotherapy of the central nervous system. We present the results of a retrospective study involving 100 patients treated between 1964 and 1971. Pretreatment bone-marrow smears from these patients were reviewed. We could distinguish morphologically 4 varieties of ALL: prolymphoblastic, macrolymphoblastic, microlymphoblastic, and prolymphocytic. For microlymphoblastic and prolymphocytic ALL, the actuarial curves of duration of the first remission reached a plateau at 62 and 51%, respectively, for patients <15 years old and 57 and 46%, respectively, for all patients studied. The actuarial curves of survival reached a plateau in macrolymphoblastic ALL as well. This difference between the curves of duration of the first remission and of survival indicates the relative sensitivity of microlymphoblastic and prolymphocytic ALL to immunotherapy and the sensitivity of macrolymphoblastic ALL to only chemotherapy. Prolymphoblastic ALL, the curves of which did not plateau, was poorly sensitive to both forms of therapy. We discuss these results in terms of choice of therapy for ALL. Long-term, intensive, "maintenance" chemotherapy seems justifiable only in patients with macrolymphoblastic and prolymphoblastic ALL, whereas patients with microlymphoblastic and prolymphocytic ALL can be treated with chemotherapy, moderate in intensity and duration, before active immunotherapy.—*Natl Cancer Inst Monogr* 35: 361–371, 1972.

THE LITERATURE is extensive on experimental active *immunoprevention* of cancer, which is the stimulation of immune reactions *before* the tumor is established. This stimulation can be specific, consisting of the administration of irradiated neoplastic cells, which generally produces a moderate effect (1, 2). It can also be nonspecific, consisting of the application of one or several agents that we have called "systemic immunity adjuvants" [see (3)]; the most widely used of these agents is bacille Calmette-Guérin (BCG) injected intravenously (2, 4-6). A marked effect is generally achieved. This stimulation can be elicited by both adjuvant and irradiated tumor cells. Though administered by different routes, adjuvants combined with irradiated tumor cells are more effective than adjuvant given alone. However, adjuvant given alone is more effective than irradiated tumor cells given alone (2).

Our present work is concerned only with *active immunotherapy*, which is the stimulation of immune reactions *after* the tumor is established. We are in urgent need of a weapon applicable to man which would be complementary to chemotherapy, since chemotherapy on disseminated tumors cannot by itself eradicate the "last cell" because it obeys first-order kinetics (7-9).

Using subcutaneously grafted L1210 leukemia as the first model to evaluate the possible effectiveness of active immunotherapy, we have shown that immune stimulation can be followed by regression of the grafted tumor (2, 10). A study of the efficiency of this treatment indicated that: 1) Systemic immunity adjuvants (BCG) given alone are rarely and, if so only slightly, effective, while irradiated tumor cells are more frequently active; systemic immunity adjuvants can potentiate the effect of the cells (even when they are given in different

sites and possibly at different times) in such a way that animals can be cured (text-fig. 1). 2) BCG only acts noticeably if given repeatedly, whereas irradiated tumor cells need not be injected more than once to act. 3) Nevertheless the effectiveness of active immunotherapy is limited, and the most important limiting factor is the number of tumor cells. Systemic immunity adjuvants, irradiated tumor cells, or both combined are only effective if the number of grafted leukemic cells is  $\leq 10^5$  (text-fig. 2) (10).

Active immunotherapy has been effective in other leukemias, *i.e.*, Rauscher and E $\phi$ K1 grafted intravenously (11). Also with L1210 [see (3)] and E $\phi$ G2 leukemias (12), it is effective if the number of grafted cells is  $>10^5$  but previously reduced by chemotherapy. Hence these experimental data indicate that active immunotherapy of leukemias is best suited for situations in which the total number of tumor cells is small, *i.e.*, the "residual disease" left after chemotherapy.

For the first clinical trials of active immunotherapy, we chose the residual disease in acute lymphoid leukemia (ALL) left after chemotherapy. However, it was suspected that this disease was an unwise choice for an attempt at immune stimulation because of a possible state of immune tolerance for the leukemic cells. Although this tolerant state had been conventionally assumed in spontaneous leukemia of AKR mice, we showed, with Doré *et al.* (13), that AKR mice can, after immunostimulation, reject an isogenic graft of spontaneous leukemia (induced by Gross virus) and can produce antileukemic cell antibodies. Oldstone *et al.* (14) confirmed this observation. Moreover, Allison (15)

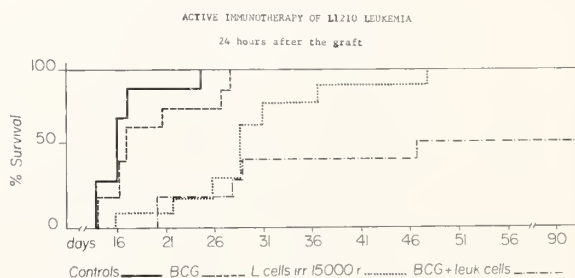
<sup>1</sup> Presented at Conference on Immunology of Carcinogenesis, held by the Oak Ridge National Laboratory at Gatlinburg, Tenn., May 8-11, 1972.

<sup>2</sup> Department of Medicine, University of California, San Francisco Medical Center, San Francisco, Calif. 94122. On fellowship year at Institut de Cancérologie et d'Immunogénétique.

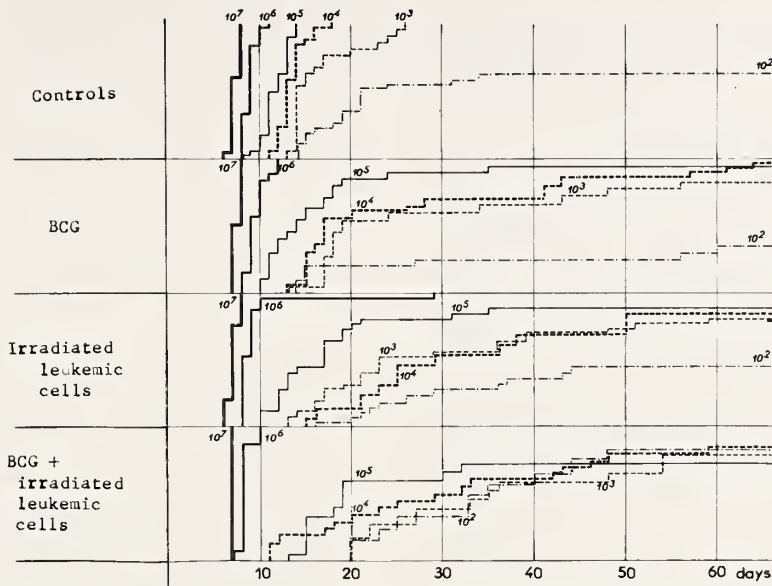
<sup>3</sup> Department of Pathology, University of Chicago, Chicago, Ill. 60637. Sabbatical year at Institut de Cancérologie et d'Immunogénétique.

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TEXT-FIGURE 1.—Cumulative survival of mice grafted with L1210 leukemia: mice not treated, mice treated with BCG (first injection 24 hours after the graft and injections repeated every 4 days), mice treated with irradiated leukemic cells (1 injection 24 hours after the graft), or mice treated with both BCG and irradiated tumor cells (2).



TEXT-FIGURE 2.—Cumulative survival of mice grafted with  $10^2$ – $10^7$  L1210 leukemia cells: untreated mice or mice treated, 24 hours after the graft, with BCG (repeated injections), with irradiated leukemic cells (1 injection), or with BCG and irradiated leukemic cells combined (10).

showed that antilymphocytic serum treatment of AKR mice shortened the latent period for the onset of spontaneous leukemia, confirming that an immunologically mediated “defense” mechanism is active in the host.

Recently, we applied active immunotherapy to spontaneous leukemia in AKR mice at 6 months of age, when leukemia is barely detectable macroscopically at autopsy. We showed that active immunotherapy can be efficient and can significantly reduce mortality (16).

An additional reason for submitting ALL patients to active immunotherapy was that Doré *et al.* (17) and Yoshida and Imai (18) detected autologous antibodies against leukemic cells in the serum of some patients. Further evidence for the immune reactivity against tumor-associated antigens in ALL patients has been the demonstration of the transformation *in vitro* of the patients’ lymphocytes stimulated by their own leukemic cells (19) and by the toxicity of the patients’ lymphocytes for their leukemic cells (20).

Further reasons for ALL being chosen for the first trial are its sensitivity to chemotherapy [which must not be confused with curability (27)] and the rarity of growth enhancement of experimental leukemias by immune stimulation (while this phe-

nomenon is seen frequently in solid tumor systems) (22).

The experiments cited above suggested that the optimum condition for the efficiency of active immunotherapy was that the patient should have the smallest possible number of leukemic cells. To achieve this condition, we reduced the cell number by chemotherapy, inducing an apparently complete remission. We then tried to further reduce tumor cell load by sequential complementary systemic chemotherapy. In addition, we administered intrathecal chemotherapy and central nervous system (CNS) irradiation because of the high incidence of meningeal relapses in ALL patients and the well-known isolation of the CNS from systemic immune reactions (23). Our recent clinical data have confirmed the efficacy of both intrathecal chemotherapy and CNS irradiation (24).

The objects of the present report were: 1) to evaluate the actuarial results of active immunotherapy in 100 patients for whom we had the diagnostic, pretherapy, bone-marrow smears and for whom the shortest follow-up was 18 months; and 2) to study retrospectively some factors influencing the effectiveness of active immunotherapy. We especially considered the influence of the cytologic varieties of ALL. We distinguished 4 varieties of



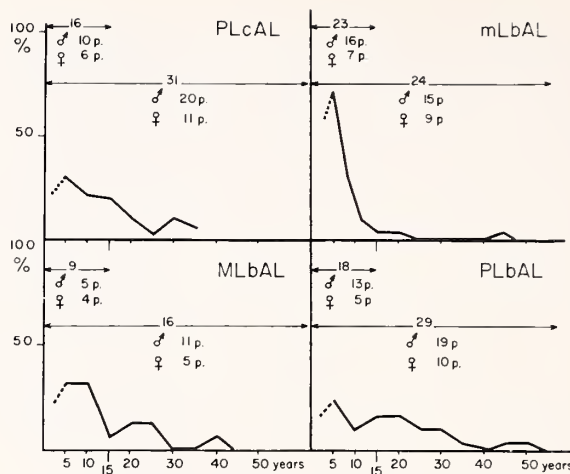
ALL: 1) polymphocytic, 2) microlymphoblastic, 3) macrolymphoblastic, and 4) prolymphoblastic [(25); see (26)].

## PATIENTS AND METHODS

**Patients.**—The criteria used to select patients were as follows: 1) The patients were treated for ALL by the protocol used by the clinical service at the time; and 2) a pretreatment bone-marrow smear was available for review and for retrospective cytologic classification.

**Classification of ALL.**—The classification was based on the appearance at the time of diagnosis of the leukemic cells stained with May-Grünwald-Giemsa. The criteria to differentiate the 4 varieties were published (25) and will be illustrated in color in a WHO monograph (26). Figure 1 illustrates the characteristics of these 4 varieties in black and white. The cells conventionally considered typical lymphoblasts were called "microlymphoblasts," when their diameter was  $<11\ \mu$ , and "macrolymphoblasts," when their diameter was larger. The cells more differentiated than the typical lymphoblasts were called "prolymphocytes"; those less differentiated than the typical lymphoblasts were called "prolymphoblasts." The leukemias were given the names of the cells that predominated (fig. 1). This classification was made in double-blind fashion by 2 cytologists and twice by the same cytologist; reproducibility was high (86%) (27).

The age and sex of the 100 ALL patients are

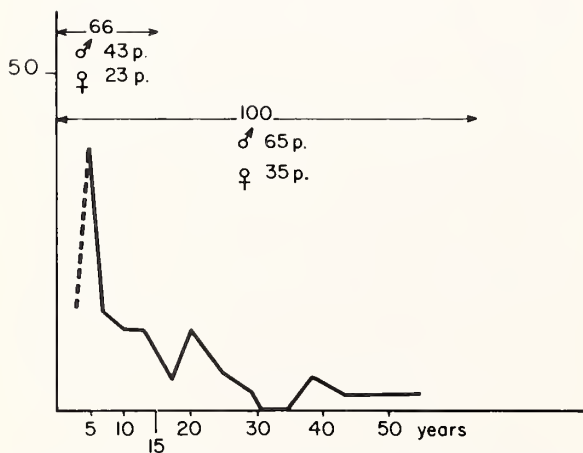


TEXT-FIGURE 4.—Relative incidence of the 4 varieties of ALL for all patients  $<15$  years old. Relative incidence in males and females. Incidence according to age. PLcAL, prolymphocytic ALL; mLbAL, microlymphoblastic ALL; MLbAL, macrolymphoblastic ALL; and PLbAL, prolymphoblastic ALL.

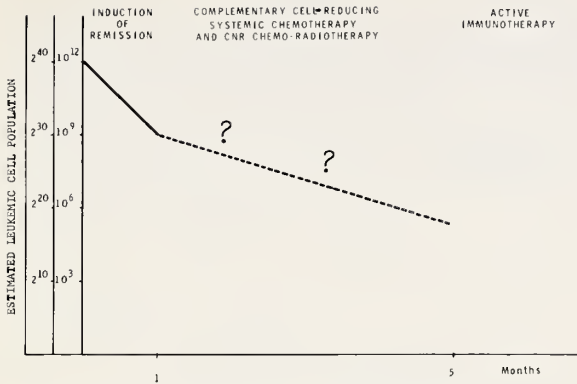
shown in text-figures 3 and 4. Also shown are the relative incidences of the 4 cytologic varieties of ALL and the age and the sex of the patients in each variety. The incidence peak at 5 years of age was more marked for the microlymphoblastic variety than for the other varieties; but it existed for all. Most patients with the microlymphoblastic variety were  $<15$  years old.

**Protocols for active immunotherapy and preceding chemoradiotherapy.**—The principle of the protocols was the same for the 100 patients (text-fig. 5). The protocols comprised 3 periods: 1) remission-induction chemotherapy; 2) complementary cell-reducing chemoradiotherapy, with chemotherapy being systemic and intrathecal and radiotherapy being applied to the entire CNS; and 3) active immunotherapy.

Since 1964 when we started this research, the protocols, while respecting the stated principle, have evolved. We have given our patients the benefit of new drugs, and the new knowledge of the value and most efficient methods of administration of these drugs. With regard to chemotherapy, text-figure 6 shows 2 kinds of protocols used for this group of 100 patients. In protocol 4, chemotherapy was short ( $2\frac{1}{2}$  months) but intensive. It consisted of repeating 3 times the remission-induction chemotherapy (prednisone, vincristine, and daunorubi-



TEXT-FIGURE 3.—Population of 100 ALL patients according to age and sex.



TEXT-FIGURE 5.—Principle of the treatment protocols.

cine). In all the other protocols, complementary cell-reducing chemotherapy was longer (5–6 months for protocols 6–8; 7–8 months for protocols 3 and 5; and 24 months for protocol 1); it was less intensive, since only 1 (protocols 1, 3, and 5) or 2 drugs (protocols 6–8) were given at a time and at doses adopted to avoid fatal toxicity.

Intrathecal chemotherapy varied from 5 injections of methotrexate (5 mg/injection) to 18 injections of methotrexate combined with cytosine arabinoside (10 mg/injection).<sup>6</sup> Radiotherapy consisted of the application of 1000 rads (protocols 1 and 3–6) to 1500 rads (protocols 7 and 8) to the entire CNS.<sup>7</sup>

Active immunotherapy consisted of specific stimulation by irradiated tumor cells and nonspecific stimulation by BCG applications combined for protocols 5–8 with injections of *Corynebacterium parvum* or *C. granulorum*; in protocols 7 and 8, poly:IC was also added as a third component. The first 3 of these agents were provided by the Institut Pasteur, and poly:IC was provided by the Laboratoire Choay. The ability of these agents to stimulate immune reactions was verified in our screening system in mice (22, 28).

BCG was applied by skin scarifications every 4th day, for the 1st month, and then on every 7th day. Twenty cutaneous scratches, each 5 cm long, were arranged in a square. Two ml of a suspension, containing 75 mg/ml of living bacteria, was applied to the scarified area.

<sup>6</sup> Our recent analysis showed that this combination adds no benefit to methotrexate alone as far as the incidence of meningeal relapses is concerned (24).

<sup>7</sup> This radiotherapy significantly benefited intrathecal chemotherapy (24).

*C. parvum* and *C. granulorum* were injected intramuscularly at a dose of 750  $\mu$ g in children and 1500  $\mu$ g in adults once a week.

Poly:IC was injected intravenously at a dose of 1 mg/m<sup>2</sup> daily for 1 month.

Irradiated allogeneic tumor cells were injected intradermally each week for 3 months and then each month at a dose of  $4 \times 10^7$ . These cells were pooled from the peripheral blood of leukemic patients, and specifically excluded were the leukemic cells of the recipient. These cells were prepared from circulating blood by leukopheresis and stored at  $-70^\circ\text{C}$  in dimethyl sulfoxide. During the first 6 injections, the cells were treated with a 4% solution of formaldehyde to inactivate a hypothetical virus; for the ensuing injections, the cells were irradiated *in vitro* with 4000 rads.

The patients who had a relapse under immunotherapy were treated again according to the complete protocol being evaluated on the service at the time of their relapse. In other words, they were re-submitted to the remission-induction chemotherapy, then to the complementary chemoradiotherapy, and then to active immunotherapy.

## ACTUARIAL RESULTS

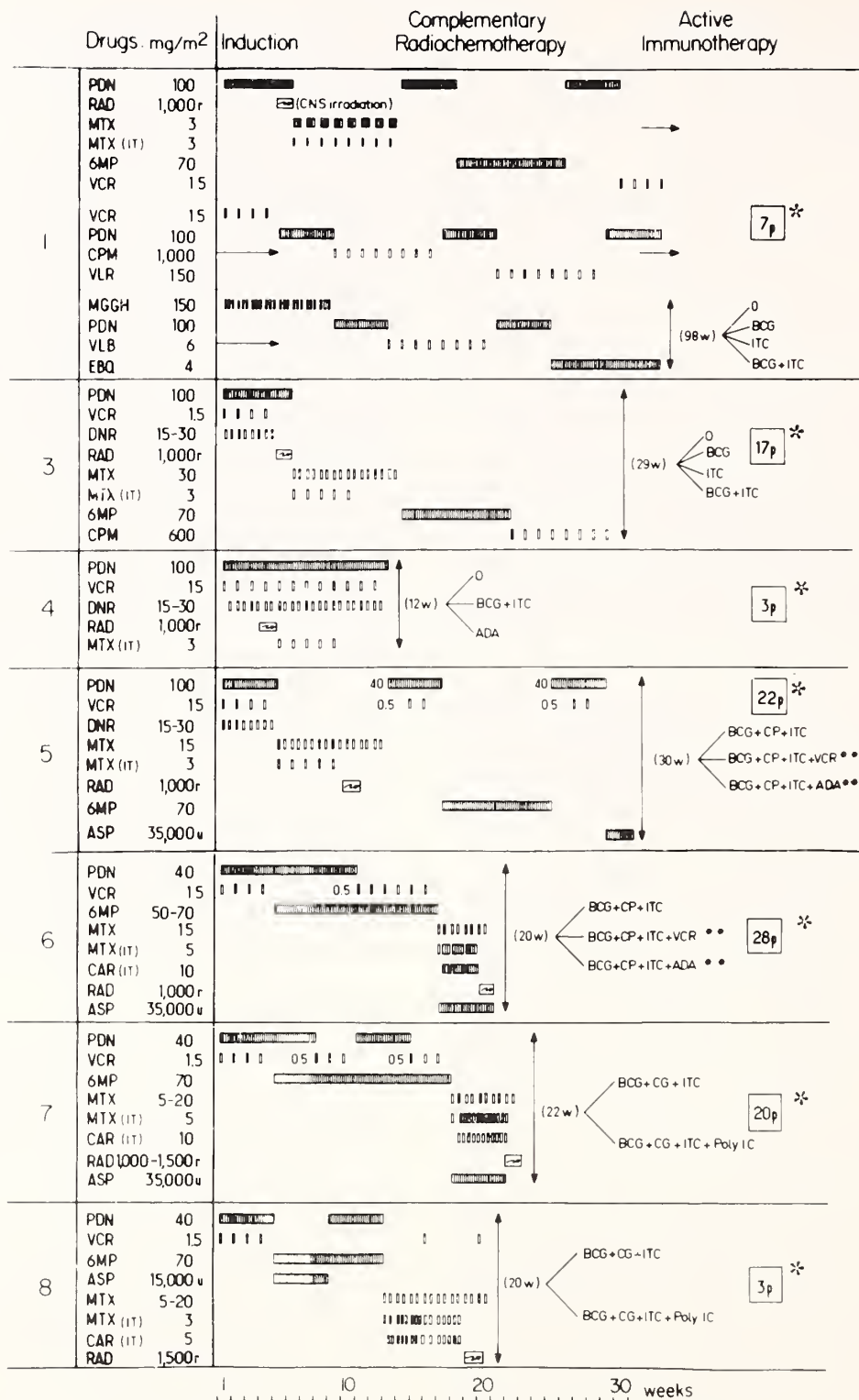
The results of these trials have been judged by the actuarial curve of cumulative duration of the first apparently complete remission and of the cumulative duration of survival. All curves are presented with the necessary geometric time scale.

## Overall Results

Text-figure 7 shows that, for patients of all ages, the median cumulative duration of the first apparently complete remission was 12 months; the median length of cumulative duration of survival was 30 months. Most interesting was the observation that these curves, after descending, broke at about the 32d month and then reached a plateau. The fraction of the patients being represented by this plateau in the cumulative duration of the first apparently complete remission was 33%. This was a statistical expression of "cure expectancy."

## Results According to Age

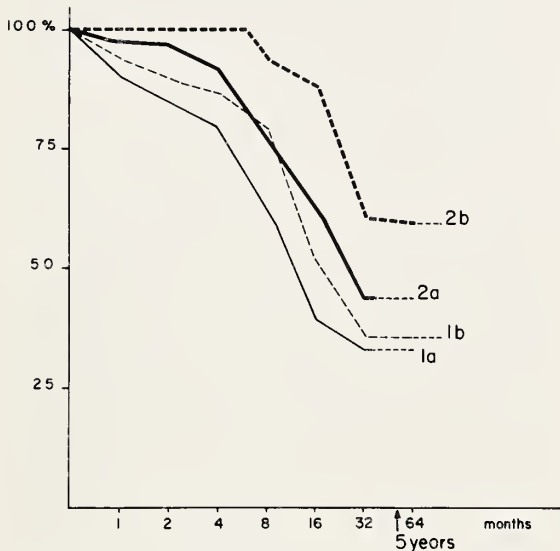
The median cumulative duration of the first apparently complete remission for the patients <15



TEXT-FIGURE 6.—Different protocols to which the 100 patients of the present study were subjected. IT = intrathecal; ITC = irradiated tumor cells; PDN = prednisone; RAD = irradiation; MTX = methotrexate; 6MP = 6-mercaptopurine; VCR = vincristine; PDN = prednisone; CPM = cyclophosphamide; VLR = vinleurosine; MGGH = methylglyoxal-bis(guanyldiazotane); VLB = vinblastine; EBQ = ethylene-imino-benzo-quinone; DNR = daunorubicine; ASP = L-asparaginase; CAR = cytosine arabinoside; ADA = adamantidine; CP = *C. parvum*; and CG = *C. granulorum*. \* = Number of patients for whom diagnostic bone-marrow smears were available to be reviewed for this study. For doses, see (30).



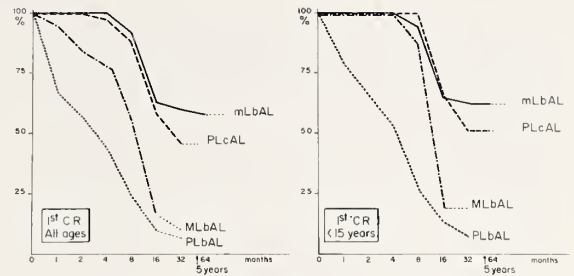
years old was 16 months, and the fraction of the patients demonstrating the plateau phenomenon was 43% (text-fig. 7). The curve of cumulative duration of survival has not yet fallen to the 50% level. The prognosis, therefore, was significantly better in patients <15 years old than that for the total population of patients.



TEXT-FIGURE 7.—Actuarial curves 1) of duration of first apparently complete remission and 2) of length of survival a) for all ages and b) for patients <15 years old. Note time scale is geometric.

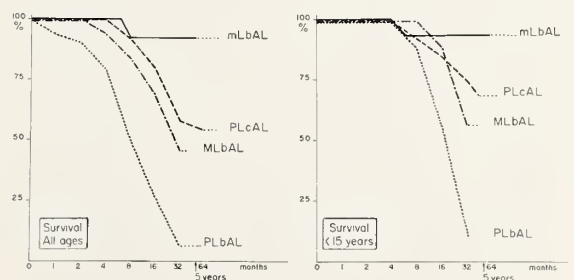
### Results According to Cytologic Varieties

The most interesting observations concerned the variations in the cumulative duration of the first apparently complete remission and in the cumulative duration of survival according to the cytologic varieties. While the curves for the cumulative duration of the first apparently complete remission of the patients with prolymphoblastic ALL and of those with macrolymphoblastic ALL descended regularly to a very low percentage at 16 months, those of the patients with microlymphoblastic ALL and patients with prolymphocytic ALL broke between 16 and 32 months and reached a plateau in 57% of the former and in 46% of the latter (text-fig. 8). Text-figure 8 also shows that this plateau was slightly higher in patients <15 years old (62% for the microlymphoblastic type) than that in the total patient population.



TEXT-FIGURE 8.—Comparative cumulative total duration of first remission (C.R.) of the different cytologic varieties of ALL (all ages and patients <15 years old) (actuarial curves). Note time scale is geometrical. mLBAL, microlymphoblastic ALL; PLcAL, prolymphocytic ALL; MLbAL, macrolymphoblastic ALL; and PLbAL, prolymphoblastic ALL.

An unexpected observation is presented in text-figure 9, which shows the actuarial curves of cumulative duration of survival. For the patients of all ages and for the patients <15 years old, there was a plateau representing about 50% of the population, not only for the two preceding varieties, but also for one of the other two, *i.e.*, the macrolymphoblastic variety. This difference between cumulative duration of the first apparently complete remission under immunotherapy and cumulative duration of survival suggests that, while the microlymphoblastic and the prolymphocytic varieties are immunotherapy sensitive, the macrolymphoblastic is immunotherapy insensitive but is chemotherapy sensitive. This interpretation is supported by the fact that, according to our protocol, a patient who had a relapse under immunotherapy was submitted again to frequently successful remission-



TEXT-FIGURE 9.—Comparative cumulative survival of the ALL patients according to the cytologic variety (all ages and patients <15 years old) (actuarial curves). Note time scale is geometric. mLBAL, microlymphoblastic ALL; PLcAL, prolymphocytic ALL; MLbAL, macrolymphoblastic ALL; and PLbAL, prolymphoblastic ALL.

induction chemotherapy, then to preimmunotherapy chemotherapy, and finally to immunotherapy.

## DISCUSSION

Before drawing conclusions from these data, we must critically evaluate the methodology. These 100 patients were treated successively by several protocols. Protocols were changed to answer specific question(s), to introduce new agents or new modalities of administration demonstrated clinically or experimentally by us or by others as being more effective, or to eliminate a form of therapy shown to be detrimental. The experience with each protocol, however, has taught us many things about the response of ALL to therapy which we carried over to the succeeding protocols.

Protocols 1, 3, and 4, for example—in which some patients were treated with active immunotherapy after sequential, long-term chemoradiotherapy and other patients, serving as controls, were given no treatment after the identical chemoradiotherapy—demonstrated that active immunotherapy was effective against ALL (29). Protocols 5 and 6 demonstrated that supplementary cytostatic or antiviral chemotherapy, given simultaneously with active immunotherapy, severely reduced the effectiveness of active immunotherapy (30). In protocol 8, the combination of asparaginase and methotrexate, used also in protocols 6 and 7, was eliminated because we showed in L1210 leukemia that this combination was less effective than methotrexate alone (31). Some protocols were abandoned early because we thought that new information would enable us to design a potentially better protocol. We can also abandon a protocol deliberately because primarily we must act ethically as physicians and secondarily we must adhere to the scientific method as best we can. On the other hand, this scientific imperfection of a retrospective study conducted with patients treated by different protocols has a saving feature because the principle behind all the protocols was uniform and unique. It consisted of the use of active immunotherapy for residual disease after complementary cell-reducing chemoradiotherapy. With regard to the correlation between the therapeutic results and the cytologic varieties, the objections above were less valid. First, the only cases classified were those in which we could re-evaluate the bone-marrow smears taken before any therapy. Then, the re-evaluation was made by 2 cytologists in a strictly double-blind

fashion. Finally, one of us (G.M.) reviewed each slide on 2 separate occasions, again in a double-blind fashion. Only then did we analyze, retrospectively, the response to therapy.

The following facts are evident from the data we have accumulated over the past 8 years.

1) A fraction of the ALL patients (33% for all ages, 43% for subjects <15 years old) are still in apparently complete remission. The curve of cumulative duration of the first apparently complete remission for these patients broke at about 32 months and continues as a plateau.

2) There is a noticeable difference in the existence of this plateau according to the cytologic varieties we have proposed. The curves of cumulative duration of the first apparently complete remission reached a plateau for a high percentage of patients for only 2 cytologic varieties, microlymphoblastic and polymphocytic. The percentage was especially high for the microlymphoblastic type, which is more frequent in children (text-fig. 3). The fact that the curve of cumulative duration of the first apparently complete remission did not reach a plateau for the macrolymphoblastic variety, while the curve of cumulative duration of survival for this same variety did reach a plateau, strongly suggests that this variety is immunotherapy insensitive but chemotherapy sensitive.

We can draw several conclusions from these data and use the information presently available to formulate a useful basis for therapy and prognosis. The cytologic correlation is very important for determining prospectively the therapeutic protocols. Since 1964, we have been treating all our ALL patients by remission-induction chemotherapy, followed by complementary cell-reducing chemoradiotherapy, and finally by active immunotherapy. This complementary cell-reducing chemotherapy has been moderate in intensity and duration, except for one trial. It has not caused, by itself, "therapeutic deaths." Other groups of investigators have been submitting patients to very intensive and/or very long, so-called "maintenance chemotherapy." The accidents and deaths attributable to such vigorous chemotherapy are not rare, being 10% in some reports (32) or more (personal communication).

It seems likely, therefore, that the choice between protocols comprising moderate complementary chemotherapy, followed by immunotherapy, and protocols comprising intensive and/or long-term chemotherapy must no longer be determined

by the geography of the patients or their doctors but by the cytologic variety of the patient's disease. While it seems sound to treat the microlymphoblastic and prolymphocytic varieties with moderate chemotherapy followed by immunotherapy, it seems reasonable to risk a more intensive and longer chemotherapy for only the other 2 varieties, especially macrolymphoblastic.

Weighing benefit and risk and on the basis of the information we have to date, we can justify planning the intensive and long-term maintenance chemotherapy only in patients with macrolymphoblastic and prolymphoblastic ALL in whom immunotherapy has not yielded the promising results indicated by the plateau feature of the curves in the cumulative duration of the first apparently complete remission seen in the microlymphoblastic and prolymphocytic varieties. For patients with the latter varieties, we maintain as our goal active immunotherapy after complementary cell-reducing chemoradiotherapy moderate in intensity and duration and minimal in risk. Further, we are justified in continuing to administer chemotherapy and radiotherapy in tandem to the CNS in all patients with ALL because our data indicate that this treatment reduces the incidence of clinical meningeal leukemia in patients subsequently treated by immunotherapy (24).

We can also extract from these data the prognostic value of this sophisticated but technically simple classification. As indicated by text-figure 9, seeing on the pretherapy bone-marrow smears mainly microlymphoblasts allows us to say to the family of the patient that there is about a 90% chance that he will be alive in 5 years. This variety of ALL is therefore one of the best cancers as far as prognosis for 5-year survival is concerned.

We know from Burchenal's study (33) that the exceptionally long-term survivors, after conventional treatment, may still have a relapse after 5 years. Though we have had no patients who have had relapses after 5 years, we feel it is still too early to know if immunotherapy will avoid such very late relapses, and at the present time the plateau phenomenon can only be interpreted as an expression of "cure expectancy."

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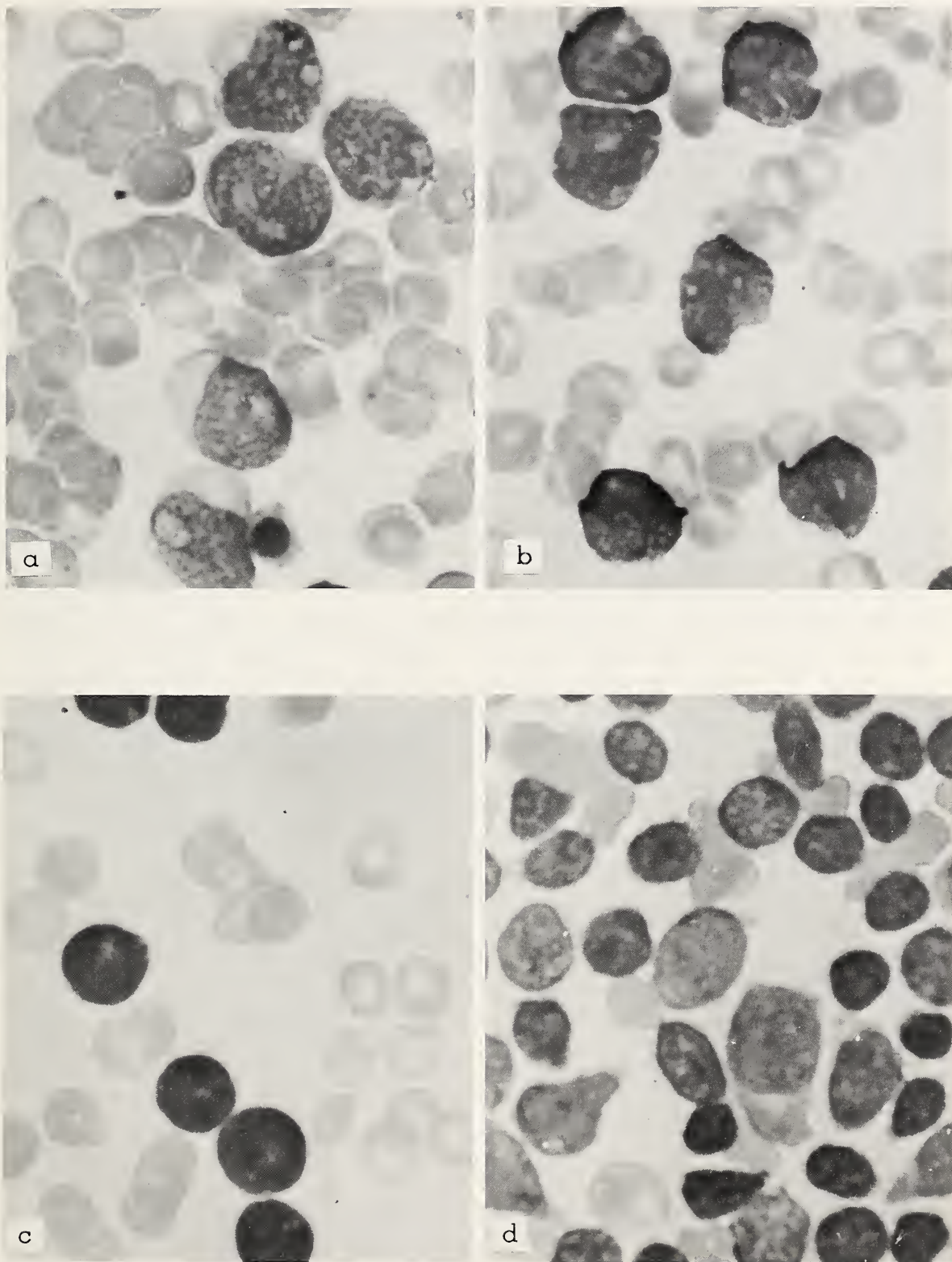


FIGURE 1.—Morphologic aspects of the 4 varieties of ALL: *a*) prolymphoblastic; *b*) macrolymphoblastic; *c*) microlymphoblastic; and *d*) prolymphocytic.





## DISCUSSION

**E. Klein:** I would like to point out that Dr. Mathé's presentation introduces a very important point: We have to be careful about what we attempt to do with chemotherapy. It is one of the holy cows that we are about to invade! And I am opening the floor to discussion with these words.

**N. Haran-Ghera:** Dr. Mathé, you started by saying something very fundamental: You think that the AKR mouse is the best model for studying leukemia. We have been criticized for years by medical people who say that all of us working with animal leukemia are using an irrelevant model for human leukemia. I think the evidence shows that the AKR leukemia is really a T-cell leukemia, whereas the human lymphatic leukemia is a B-cell leukemia—

**G. Mathé:** The chronic.

**Haran-Ghera:** Not the acute?

**Mathé:** No, only chronic.

**Haran-Ghera:** I'm sorry. That is what I was told; therefore, I thought that maybe we really should look for a different model.

**R. F. Barth:** Dr. Mathé, according to the classification system you have developed, do you see any changes such as macrolymphocytic reverting to the microlymphoblastic form, or from prolymphocytic?

**Mathé:** First, this classification is made on smears. It is difficult to classify because after that it changes. The reproducibility has been very good in 2 different blind studies. In one, it was myself versus myself; in the other, it was myself versus a technician. The reproducibility was good except between prolymphoblastic and macrolymphoblastic in which we differed in 20% of the specimens examined.

**J. Stjernswärd:** Dr. Mathé, have you found any evidence of immunoglobulin coating on your cells? If so, have you found any difference between the microlymphocytic and the prolymphocytic forms as compared to the resistant macrolymphocytic form?

**Mathé:** These are under study. It is a very difficult study, and the patients who do the best are those who have fewer cells in their bone marrow. There is a competition between the cells for treatment and the cells for study. But this is under study for cell-mediated reactions and humoral immune reactions.

**Stjernswärd:** Regarding the mechanics as Dr. Zbar demonstrated, close contact would be necessary if a cell-mediated reaction occurs. Alternatively, humoral factors, possibly interferon, may be involved. Have you studied the effect of bacille Calmette-Guérin (BCG) on interferon?

**Mathé:** You mean with BCG? No, we have been studying the effects of poly I:C, and we have not produced interferon with 1 mg of poly I:C for 30 days, which acted as an immunostimulant, but not as an interferon inducer.

**C. E. Mawas:** I would like to have some clarification on your protocol for leukemia. You report 5-year survival. When did you start immunization with BCG alone? When did you introduce BCG plus cells? When did you introduce the new protocol? And which of your reported cases of survival could be really compared to the acute leukemia

group B? How many patients were there? How many were randomized? Was all the chemotherapy stopped at 5 months?

**Mathé:** The first protocol comprised 30 patients: 10 controls and 20 patients receiving immunotherapy. In those 20 patients given immunotherapy, there were 3 subgroups. In one subgroup we did not see a difference between controls and study patients. In another subgroup we had all the experimental data and all of them were given BCG and tumor cells. The results are still the same; they have not been affected. Then we started a new protocol. In the old protocol, chemotherapy was not discontinued while immunotherapy was used, and the approximately 100 patients I was speaking about today were able to be reviewed for the first time. This is what we found in that group of about 100 patients: The duration of first remission is the duration of remission after its induction, and the duration of survival is the duration of survival from the beginning of the disease. In the immunized patients we have some meningeal relapses without bone marrow relapses. I don't know if we have more than the chemotherapist. This has not been studied and should be studied.

**G. W. Santos:** Dr. Mathé, do you have any evidence that the tests we have heard about in the past few days have any correlation, because we have talked about these for 2 days? We have seen your results here, and we would be encouraged if there are some *in vitro* tests that would predict those patients who are going to do well.

**Mathé:** Presently, we don't have enough data because we don't have enough cells. We hope to replace the cells preserved at low temperature by cultured cells; the cells which we get from leukemia patients, or from those we think may be leukemic, are being cultured as well as used for therapeutic studies. We will have more cells at the beginning to preserve for the later study. But at the present time we feel strongly that we must use most of the cells for therapy. Then the tests, however, are ready. We have some data on the patient who has a lot of cells, but we don't have enough data on the patient who has only a few leukemic cells.

**J. Gutterman:** I would like to emphasize a very important point to which Dr. Mathé alluded in passing: Blocking may occur in leukemia, but enhancement rarely, if ever, occurs. We have studied a series of 35 adult acute leukemia patients in the past year and in 8 of 24 patients with acute myeloblastic leukemia (AML) but in 0 of 11 patients with acute lymphoblastic leukemia (ALL) we have seen complete or partial abrogation of the blastogenic response in autologous serum but a vigorous response in normal allogenic serum. In 7 of 8 AML patients with blocking, we have demonstrated significant coating of the leukemia cells with IgG. In contrast, only 1 of 16 AML patients without blocking effect and 0 of 11 ALL patients had coating of leukemia cells.

These AML patients, in contrast to solid tumor patients with blocking of cytotoxicity as described by the Hellströms, have had a good prognosis. All but one have had chemo-

therapy-induced remissions, and all but one of the latter have remained in remission for 3+–12+ months. So, when we talk in terms of blocking and immunoglobulin coating of tumor cells, we really have to make a very clear distinction between solid tumors and the leukemias. Humoral immunity may play a very important role in the leukemias.

I have one more specific question. How viable are the allogeneic cells that you give? Are these 90 or 100% viable?

**Mathé:** At the beginning we preserved allogeneic cells at  $-70^{\circ}\text{C}$  and viability wasn't very good. Now we preserve them at  $-190^{\circ}\text{C}$  and viability is much better. We also irradiate them with 4000 rads.

**M. M. Black:** In 1947, I published a paper on energetic mechanisms in tumor cells, particularly leukemic cells, and in that paper was a picture of large blasts and small blasts as they occurred in the same case, and these did change. The point I suggest to you is that, since these cells are metabolically quite distinct and have different energetic pathways, it might help to check this point *in vitro* in your assessing for chemotherapy.

**Klein:** Thank you, Dr. Black.

**Mathé:** We are now studying the possible differences, as far as kinetics or antigenicity is concerned, or the possibility of growing these cells in culture, and so on, but we don't know yet what the difference is. We know what the rate of reproduction is, and there is good correlation with prognosis.

**D. M. Mumford:** In view of the fact that it is so difficult to obtain cells for immunization—and we followed your protocol on some patients—would some of your results be due to the fact that the patients, if you are using irradiated cells, might come from either your micro-macroplasma group? In other words, you have to take the high-cell-count type of patients. Have you studied this aspect, since it would be important for the immunization procedures that you are using to classify your irradiated cells?

**Mathé:** We did not know this classification when we started. We used a pool of cells, which makes it impossible to classify the cells we used in earlier studies.

**Mumford:** But you had to use the high-count patients to get enough blood to use those cells.

**Mathé:** We usually use the cells of patients who have high counts, and the patients who have high counts are usually polymphocytic patients.

**A. LuBuglio:** In looking at the problem of how cellular immunity is maintaining remission, we can also learn something from the failures. For example, we have studied one female patient who had been on a BCG protocol for 4 months. This patient had a very strong skin reaction to

BCG and had positive migration-inhibition factor (MIF) *in vitro* to purified protein derivative (PPD). She developed a brief, 48-hour, mild illness which was viral meningitis. During the subsequent week, she was found to be allergic to PPD by skin test. She did not make MIF to PPD *in vitro*. She had a perfectly normal bone marrow and peripheral blood at that time, but had a relapse 7 days later of her leukemia. She subsequently received just one dose of vincristine, and 10 days later she was in complete remission, coinciding with the fact that her original PPD skin tests and BCG tests flared and became positive. We may find that a number of people who are on these regimens will have a relapse when concurrent illnesses modify their cellular immune response. We must study our patients very closely in terms of what incidents occur around their relapses.

**Mathé:** We have seen some patients whose BCG reactions were becoming negative before their relapse, but we have seen some who had positive BCG reactions and still had a relapse.

**Klein:** Dr. Mathé, do you monitor the immunocompetence of your patients before you put them on the protocol?

**Mathé:** Yes.

**Klein:** And how do you do that?

**Mathé:** I said that the tests are not very good. We have—you mean nonspecific?

**Klein:** Skin tests.

**Mathé:** Yes. We use skin tests to various antigens including BCG. Since the patients are on chemotherapy, they are often negative in the beginning. When we finish up what is called intermittent chemotherapy, the skin tests become positive again. This is the time when we start immunotherapy. But we don't leave any gaps between the end of chemotherapy and the beginning of immunotherapy, and this may be a big mistake.

**Klein:** It should be pointed out that if the patient is not immunocompetent, a response cannot be expected. Their data should be excluded from your study because you are just weighing it unfairly.

Have you had any experience with the high methotrexate citrovorum rescue method introduced by Dr. Djerassi in which immunocompetence is more rapidly reestablished than during conventional chemotherapy?

**Mathé:** No, but I think any intermittent chemotherapy causes less interference with the immune action than continuous chemotherapy. But since we do not know the kinetics, we do not know exactly the right time to apply immunotherapy after the last shot of chemotherapy.

**Klein:** Thank you very much, Dr. Mathé.

# Immunotherapy of Human Melanomas and Sarcomas<sup>1, 2</sup>

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**SUMMARY**—At present, immunotherapy should be regarded as a method of therapy that can be useful in treatment of selected patients with malignant melanoma. Preliminary evaluation of a method of immunotherapy of proved effectiveness, with a model system developed from study with a guinea pig sarcoma, has been encouraging—an enhanced immune response can be induced in patients with melanomas and sarcomas after immunization with irradiated tumor and bacille Calmette-Guérin. We are presently evaluating this procedure in patients who have recurrences after definitive cancer surgery or who have a high likelihood of developing recurrent disease. With these patients, we hope to find the optimal conditions for immunotherapy in man. When such conditions are defined and the safety and effectiveness of this method of treatment are established, it is likely that immunotherapy will prove useful as an adjunct to cancer surgery, radiation therapy, and chemotherapy.—*Natl Cancer Inst Monogr 35: 375–378, 1972*

EVIDENCE accumulated during the past 5 years clearly indicates that human neoplasms contain tumor-associated antigens not found in normal adult human cells. These tumor antigens have been detected primarily by their ability to elicit humoral and cellular immune responses in cancer patients. Sensitive immunologic techniques used to demonstrate these findings include: immunofluorescence (1, 2), colony inhibition (3), complement fixation (4), immunodiffusion (5), complement-dependent cytotoxicity (6), lymphocyte transformation (7), and delayed cutaneous hypersensitivity reactions (8).

Can the patient's immune response to these tumor-associated antigens influence the course of his malignant disease? Previous studies from our lab-

oratory (4, 9) suggest a remarkable correlation between the antitumor antibody titer determined by complement fixation and the clinical course of malignant disease in melanomas and sarcomas.

When serum samples, obtained from patients with skeletal and soft-tissue sarcoma at various stages of their clinical course, were assayed by the microtiter complement-fixation test for antisarcoma antibodies, it was found that patients who enjoyed long-term survival from treated sarcomas showed a persistently elevated antisarcoma antibody titer. The persistence of antibody varied only slightly up to 3–4 years after removal of the primary sarcoma. When sequential serum samples were obtained from patients before and after surgery for primary sarcoma, all patients who had definitive surgery and continued to be free of disease had at least a fourfold rise in antitumor antibody after removal of the tumor. These antibody titers remain elevated to the present time in those patients who remain free of disease.

In contrast, patients who had no antisarcoma antibody before surgery had no increase in anti-

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body titer after surgery, and all but one patient developed recurrence within 6 months. Furthermore, of several patients who demonstrated low titers of antibody before surgery and had a transient rise in antibody titer after tumor removal, but who developed pulmonary metastasis, all showed progressive decline in their antisarcoma antibody titers to nondetectable levels as their metastatic disease progressed.

A smaller number of melanoma patients were studied, and a similar correlation was also found between antibody titer by complement fixation and clinical course of their malignant disease (10).

The remarkable correlation between antitumor antibody titer and the course and extent of disease in patients with malignant melanoma and skeletal and soft-tissue sarcomas suggested to us that the antigens involved could be important for the patient's host-immune response against his tumor. Therefore, it seemed logical to try to increase the patient's immune response against these antigens with immunotherapy.

Before immunotherapy of human neoplasms was attempted, however, experiments were performed in inbred guinea pigs with liposarcomas induced by 3-methylcholanthrene (MCA-A). The experimental model was similar to that recently described by Kronman and associates (11). In these experiments, living tumor cells were injected intramuscularly into the leg of a guinea pig; immunotherapy was then instituted up to 10 days later by multiple intradermal inoculations of lethally irradiated tumor cells mixed with bacille Calmette-Guérin (BCG) vaccine. (BCG was used as an immunologic adjuvant in these studies because it will heighten immune response against a wide variety of animal neoplasms.)

This method of immunotherapy significantly inhibited the growth of the intramuscularly injected tumor (4). Blood samples from these guinea pigs also demonstrated a striking rise in antibody titer against tumor-specific antigens of the guinea pig sarcomas when sera were tested with the complement-fixation test (12).

We were encouraged toward clinical trials of immunotherapy in man as a result of these studies with guinea pig sarcomas. To date, 40 patients have been studied—25 with malignant melanoma and 15 with skeletal and soft-tissue sarcomas. The results of immunotherapy in patients with these 2 diseases will be discussed separately.

All 25 patients with malignant melanoma had advanced disease with multiple subcutaneous or intradermal metastatic melanoma nodules, as well as metastases to parenchymal organs in some cases. BCG was injected directly into the cutaneous metastatic melanoma nodules (13). Approximately 90% of the melanoma nodules directly injected with BCG regressed in 19 patients who were immunologically competent, as judged by their ability to be sensitized to dinitrochlorobenzene (DNCB), a potent agent for inducing delayed cutaneous hypersensitivity (14). In addition, 20% of those patients who had nodules at sites peripheral to the BCG inoculations showed regression of these uninjected nodules as well; they have had remission of their disease for as long as 4 years. In contrast, only 1 of 6 patients who could not be sensitized to DNCB had regression of his lesions after injection with BCG. This patient was anergic to DNCB, though he did react positively to tuberculin tests after BCG therapy, thus indicating that he did have some cellular immune response to the BCG. Immune response in these patients was associated with a rising titer of antimelanoma antibodies. Sequential biopsies of tumor nodules after BCG inoculation revealed regression of these nodules was associated with a granulomatous infiltration of lymphocytes, monocytes, and fibroblasts that surrounded and infiltrated the melanoma cells.

The mechanism for tumor regression after immunotherapy with BCG in these patients is unclear. BCG had no direct antitumor effect, since it did not cause tumor regression in tuberculin-negative patients. Specific and nonspecific immune reactions were probably involved. BCG is known to be a potent immunologic adjuvant capable of increasing host immune response to a wide variety of tumor-specific antigens in animal neoplasms. Nevertheless, the fact that the observed regression of melanoma nodules occurred after direct injection of the nodules only in tuberculin-positive patients suggests that a large part of the antitumor effect was nonspecific and resulted from the delayed-hypersensitivity reaction occurring within the melanoma nodule. However, a specific immune response to the melanoma antigens also occurred in some patients, since the appearance of lymphocytic infiltrates with regression of uninjected melanoma nodules observed in these patients coincided with a rising titer of antimelanoma antibodies. Apparently, then, BCG has no direct antitumor effect,

since it did not cause tumor regression in any patient who did not become tuberculin positive after BCG therapy.

Various complications can develop after immunotherapy with BCG vaccine, the most frequent being ulcerations at the site of BCG inoculation that require 2–3 months to heal. Fever spikes and nausea were seen shortly after multiple inoculations of the vaccine into patients who were previously treated with BCG, and one patient had a transient anaphylactoid-type reaction. Three patients had evidence of systemic BCG infection with prolonged fevers, hepatosplenomegaly, abnormalities in liver function tests, and other evidence of systemic infections. Two of these patients had spontaneous relief of symptoms without antitubercular therapy. One patient developed a severe systemic infection that has required prolonged drug therapy. It seems likely that the risk of such infections with BCG therapy will be greater in patients whose immune responses are depressed, as indicated by their inability to become sensitized to DNCB.

Immunotherapy studies have been undertaken in 15 patients with skeletal and soft-tissue sarcomas in an effort to alter their immune response to their own tumors. The immunization procedure used involved injecting multiple intradermal sites with 50–75 million irradiated tumor cells mixed with BCG vaccine, as in the guinea pig experiments.

We studied each patient's immune response by testing postimmunization serum for complement-fixing and cytotoxic antibodies and by skin testing the patient against his own autologous tumor cells.

From 2–3 weeks after initial immunizations in 9 of 12 patients studied, antisarcoma antibody titer rose dramatically from low or nondetectable levels to as high as 1000–2000. Complement-dependent cytotoxic antibody (6) also rose in the sera from 9 of 14 patients studied. Complement-dependent cytotoxic reaction was specific for the sarcoma cells, whereas antibody activity did not rise against cultures of normal fibroblasts from any patient whose sarcoma cells had undergone immune cytolysis with the postimmunization sera.

Evidence that stimulation of the cellular immune response had occurred was observed in the skin-test reactions of these patients. Positive delayed cutaneous-hypersensitivity reactions to autologous tumor cells, which were not present before immunization, developed in 7 of the 15 patients. Thus both the humoral and cellular immune response to

human sarcomas could be augmented by immunization of patients with their own neoplasms, with BCG as an immunologic adjuvant.

The therapeutic results of this clinical trial of 15 patients cannot be evaluated adequately at present. They may be briefly summarized as follows: The 5 patients whose metastatic pulmonary disease could not be entirely resected before the institution of immunotherapy have shown no significant clinical response. Of the 5 patients with bilateral pulmonary metastatic disease, in whom all gross metastatic lesions could be resected, 2 have shown some evidence of improvement in their clinical course. One patient is free of disease 2 years later. Another patient died 14 months after immunotherapy was discontinued. On the basis of tumor-doubling times and the known growth patterns of their disease before immunotherapy, we think survival was prolonged in these patients significantly over that which could be expected by surgical resection of their metastatic disease alone.

Immunotherapy was initiated in 5 patients after resection of their primary sarcoma before the development of metastatic disease. Further follow-up is necessary to evaluate the usefulness of immunotherapy in this clinical setting. However, none of these patients who were free of metastatic disease at the time of their primary surgery have developed pulmonary metastases following immunotherapy after observation for up to 1 year.

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## Immunotherapeutic Approaches to the Management of Neoplasms<sup>1, 2</sup>

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**SUMMARY**—The principles underlying immunotherapy for skin cancer have been extended to immunotherapeutic approaches to metastatic lesions of melanoma, adenocarcinoma of the breast, and multifocal malignant diseases including mycosis fungoides, reticulum cell sarcoma, and Kaposi's hemorrhagic sarcoma. Regression of various degrees, including microscopically proved eradication of malignant lesions, was demonstrated as a result of cell-mediated immune challenge. The degrees of regressions of primary and disseminated lesions depended on the state of immunocompetence of patients. Patients with advanced impairment of cell-mediated immunity did not respond. Retention of immunologic memory in patients whose immunocompetence for recognizing new antigens had been lost was found to provide a basis for cell-mediated immune challenge responses, resulting in regressions of tumors. The concurrent use of  $\geq 2$  sensitizing agents induced regressions as a result of immune challenge; individual sensitizing agents were ineffective. Cell-mediated challenge reactions at single sites of lesions of mycosis fungoides and reticulum cell sarcoma resulted in regressions of unchallenged lesions. Combinations of immunotherapeutic approaches and chemotherapy produced tumor resolutions; each modality alone was not adequate. Immunotherapy for malignant and premalignant epidermal lesions resulted in high cure rates, as well as earlier diagnosis than otherwise possible, and was followed by a reduced incidence of new lesions in patients with previously persistent development of tumors, thus suggesting the possibility of immunoprophylaxis. Preliminary experimental evidence points strongly to monocytes and macrophages in conjunction with serum factors as being integral components of the mechanisms by which tumor cells are destroyed in association with induced delayed-hypersensitivity reactions.—*Natl Cancer Inst Monogr* 35: 379–402, 1972.

IT WAS previously shown that induction of delayed-hypersensitivity reactions at tumor sites resulted in selective reactions against premalignant

and malignant epidermal lesions which led to their eradication (1–6). Early lesions, otherwise clinically undetectable, became apparent by their response to immunologic challenge, thus permitting diagnosis and treatment. Also, differentially more intense immune reactions destroyed premalignant lesions, subsequently preventing their progression to the frankly malignant state.

In this presentation, our observations in parallel studies will be summarized which indicate that

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the immunologic principles developed in epidermal neoplasms in man pertain to broader aspects of tumor biology and apply to immunotherapeutic approaches for a spectrum of malignant diseases in man, ranging from premalignant and malignant epidermal neoplasms to lymphomas, sarcomas, melanomas, and adenocarcinomas. Our findings demonstrate that, as a result of inducing a cell-mediated challenge reaction in a relatively small area, distant unchallenged lesions, both in the skin and at other sites, may undergo immune reactions followed by regression. We will further describe immunotherapeutic approaches utilizing immunologic memory in those patients whose immunocompetence had been impaired to a degree at which cell-mediated immunity to new antigens could not be induced. Data will be presented showing that the concurrent administration of  $\geq 2$  sensitizing agents increases the antitumor activity of cell-mediated immune reactions; single agents do not. Furthermore, we have found that synergistic antitumor effects could be obtained by combining immunotherapeutic approaches with chemotherapy.

## CLINICAL INVESTIGATION

### Studies on Premalignant and Malignant Epidermal Tumors

Our initial immunotherapeutic approaches were carried out on patients with primary epidermal neoplasms. These neoplasms included multiple, extensive, premalignant epidermal lesions, such as keratoses of the surface epidermis and leukoplakia involving mucous surfaces and mucocutaneous structures, multiple intractable basal cell carcinomas, and squamous cell carcinomas.

#### *Procedure for Immunotherapy of Skin Tumors*

The initially used procedure, which has now been modified in several respects as detailed below, included the following steps:

1) The patient's level of immunocompetence is determined by skin testing.

2) Delayed hypersensitivity is induced to a small organic chemical such as dinitrochlorobenzene (DNCB). The sensitizing agent is applied in a semisolid (cream) base at a concentration of 1 part per 10,000 by a modified occlusive patch-test technique

until a hypersensitivity challenge reaction becomes manifest.

3) The sensitizing agent is titrated by serial dilution. As the concentrations are successfully lowered by 1 order of magnitude, the intensity of the challenge response decreases. The lowest level to which the patient develops a minimal delayed-hypersensitivity challenge reaction is determined.

4) A concentration 1 or 2 orders of magnitude below the lowest concentration to which a delayed-hypersensitivity challenge reaction could be demonstrated in normal skin is applied to areas involved by neoplasms.

5) Administration of preparations containing the sensitizing agent is not limited to the site of clinically detectable involvement but is extended over the anatomic region where the neoplasm is located. Thus, lesions on the forehead are treated by topical administration of the sensitizing agent to the entire forehead or face. These administrations are continued at the time the delayed hypersensitivity occurs, is maintained, and subsequently subsides, while the minimum effective concentration of the challenging agent is being administered.

6) During this period, neoplastic lesions undergo successive stages of delayed-hypersensitivity reactions including, but not necessarily reaching, necrosis. After the attainment and maintenance of a peak reaction for variable periods, involution or disappearance of tumors occurs, which is accompanied by granulation tissue formation and re-epithelialization. Thus, the site of previous tumor involvement is being resurfaced at the same time the tumor is involuting.

7) Induction of challenge reaction may be discontinued when re-epithelialization and granulation tissue formation are apparent; alternately, it may be maintained until healing is complete.

The agents used in these studies were DNCB, triethylene-imino-benzoquinone (TEIB), 5-mercaptop-2-deoxyuridine (MUDR), and several others. The concentrations for the induction of a challenge reaction ranged from 1 part to 100,000 parts of base to as low as 1 part of sensitizing agent per 1 billion parts of base.

#### *Eradication of Superficial Skin Neoplasms*

Studies of superficial epidermal tumors have included 90 patients with multiple skin cancers and premalignant lesions.

Follow-up studies on an initial group of 24 patients for periods of 5 years showed a 95% rate of eradication of neoplasms without residual tumor or recurrences in an aggregate of >5,000 lesions. The initially studied group of patients had intractable disease for which therapy by standard modalities was not adequate. The group consisted of patients with the multiple basal cell nevroid syndrome, late radiation dermatitis with malignant degeneration, xeroderma pigmentosum, arsenical dermatitis, and severe skin damage with multiple malignant lesions due to excessive exposure to the ultraviolet light of the sun or artificial sources (figs. 1-3).

Fifty-four patients with less severe disease who were studied for shorter periods were followed during the past 3-5 years, and essentially similar results were found. However, definitive evaluation in malignant disease requires a 5-year minimum follow-up observation, and the course of these patients after immune challenge has to remain under continued surveillance. Some of these patients were subsequently placed on a second study in which MUDR was investigated; MUDR was found to be a sensitizing agent in approximately 80% of a group of 22 patients.

While MUDR is an antimitotic agent, it was found not to have antitumor activity on topical administration at the doses used (0.1-0.001%) unless hypersensitivity was induced. Therefore, the antimitotic activity of the agent apparently was not a primary basis for the antitumor effect observed after its administration. These observations are of interest, since MUDR, contrary to other substituted pyrimidines, acts solely as an inhibitor of thymidylate synthetase, while other substituted pyrimidines can be converted from the deoxyribose compound to the corresponding ribosides and thus enter into RNA synthesis, producing a fraudulent RNA. The data indicate that the induction of tumor regression by MUDR is primarily due to the ability of MUDR to induce delayed hypersensitivity.

The other 37 patients who were placed in this study were the first group to be followed by a modified protocol based on the data obtained from prior exploratory studies. These patients have been observed for periods ranging from one-half year to a few months. Critical follow-up based on protocol studies will require at least an additional 3½-5 years. The data obtained so far appear to agree

with those obtained in the preliminary and ongoing studies.

The information obtained from these studies justifies the conclusion that hapten-induced delayed hypersensitivity provides a basis for effective tumor eradication with minimal recurrence.

#### *Detection of Clinically Unrecognized Neoplasms*

Selective responses to immune challenge also reveal otherwise undetectable neoplasms. As a result of the challenge response, these early tumors undergo resolution. Clearly these observations are limited by the tumor size. It is unlikely that, at an earlier stage of development, smaller tumors are also responding to challenge without a sufficient reaction to allow observation. Nevertheless, the response results in their resolution and therefore decreases the incidence of subsequent, clinically more significant neoplasms.

#### *Reduction in Incidence of Tumors*

Patients with multiple skin cancer syndromes in the normal course of their disease develop multiple tumors throughout the major part of their lives once their disease has manifested itself. Standard therapeutic procedures, surgery, including electrosurgery, cryosurgery, scapel surgery, or radiation therapy may not be the treatment of choice in view of the large proportion of the body surface involved. These procedures are usually limited to those lesions which appear to be rapidly progressive and have no effect on the subsequent incidence of tumor development. Since most patients under study had been treated by standard modalities (the only methods of therapy on hand), data are available on the incidence of new lesions in these patients, who have been closely followed in our Department.

The incidence of tumors in 7 patients with xeroderma pigmentosum, requiring as many as 100 surgical procedures per year, was reduced so that few or no surgical interventions were needed during observation periods ranging from 3-7 years. Studies at symmetrical sites, in which one side was submitted to intermittent immunologic challenge while the other side (control) was treated by standard therapeutic modalities, revealed 56 to 0 tumors, 32 tumors to 1 tumor, and 89 to 0 tumors, respectively, over observation periods of 1 year. On



reversal of the challenged and control sides, analogous data were obtained. The number of lesions following the crossover, however, was  $<10$  tumors on the control sides, presumably due to the prior eradication of vestigial lesions. Most of the lesions that arose could be managed by subsequent immunologic challenge reactions and did not require surgery, since they were not allowed to progress to more advanced stages. Data on 2 patients with the multiple basal-cell nevus syndrome also indicated the prophylactic value of the immune challenge regimen.

### **Immunotherapeutic Approaches Based on Immunologic Memory**

To induce a cell-mediated immune challenge reaction resulting in therapeutic effects on tumors, the patient has to be able to develop a hypersensitivity state against the antigen(s) used. A number of patients with multiple epidermal tumors could not develop an immune reaction to several sensitizing agents under study, which included DNCB, TEIB, para and ortho phenylenediamine, phthallic acid, picric acid, and other agents.

Studies were therefore initiated to determine whether these patients had retained immunologic memory for antigens to which they may have been exposed before impairment of immunocompetence, particularly antigens of microbial origin. These antigens included purified protein derivative of tuberculin (PPD), old tuberculin, mumps vaccine (MV), streptokinase-dornase (varidase-V), candida extract (CE), histoplasmin (H), coccidioidin (C), blastomycin (B), trichophyton (T), pertussis (P), and other bacterial and fungal antigens.

A group of 68 patients was investigated. Nine patients who failed to develop hypersensitivity to DNCB showed immunologic memory to  $\geq 1$  microbial antigens. There was considerable variation in the intensity of the challenge reactions to microbial antigens.

Immunologic memory was found by Holtermann *et al.* (7) to provide a basis for inducing selective antitumor effects by cell-mediated challenge in patients who had retained the capability of reacting to  $\geq 1$  of these antigens. Cell-mediated challenge reaction induced by microbial antigens resulted in eradication of premalignant and malignant epidermal tumors as well as cutaneous lesions of

mycosis fungoides and reticulum cell sarcoma and other nonepidermal tumors.

The methods used resembled essentially those described above for hapten-induced challenge reactions. Initially, however, microbial antigens were injected intralesionally at varying concentrations. It was found subsequently that topical administration of microbial antigens to cutaneous lesions of mycosis fungoides and other types of tumors, to be described below, was effective.

Studies by Holtermann *et al.* demonstrated that considerable variation existed in the rate and degree of responses obtained in patients with mycosis fungoides and other disseminated or metastatic tumors. Lesions in mycosis fungoides regressed after a single injection of PPD, although more usually multiple, daily injections for periods of up to 6 weeks were required, ranging from 3–42 consecutive, daily, intralesional administrations. These findings indicate a general principle of cell-mediated immunity in tumor biology. From a more practical aspect, these findings permitted immunotherapeutic approaches, based on immunologic memory for cell-mediated challenge, to be extended to a larger number of patients with extensive malignant disease with immunocompetence impaired to the extent that they could not develop *de novo* cell-mediated immunity. The data further indicate that sensitizing agents of relatively large molecular weight can produce reactions similar to those induced with compounds of small molecular weight. These observations may also be of significance in that they raise the possibility that exposure to microbial antigens as they occur throughout life serves an additional or incidental purpose of increasing the effectiveness of the immunologic surveillance mechanism.

### **Immunotherapeutic Approaches to Nonepidermal Tumors**

Antitumor effects of cell-mediated immune challenge reactions, analogous to those previously described for epidermal neoplasms, were demonstrated in several types of tumor including reticulum cell sarcoma, mycosis fungoides, Kaposi's hemorrhagic sarcoma, malignant melanoma, and adenocarcinoma of the breast (5, 7, 8). These studies further indicated that the essential principles underlying the therapeutic effects of cell-mediated immune challenge reactions, as initially observed in

malignant tumors of the epidermis, have broader implications for tumor biology and tumor therapy and pertain to a variety of malignant diseases. Regressions of lesions were induced by challenge reactions following hapten-induced immunity or on the basis of immunologic memory, or both. Responses in lesions involving the skin and subcutaneous tissue included complete regressions, partial regressions, or temporary arrest of progression, and complete lack of response, as indicated by clinical observation and serial biopsy examinations of metastatic lesions of carcinomas or melanomas and infiltrates arising from lymphomas, such as reticulum cell sarcoma or mycosis fungoides, and Kaposi's hemorrhagic sarcoma.

#### *Effects of Immune Challenge on Cutaneous Lymphomas*

Immune challenge resulted in selective reactions with and without regressions of lesions of mycosis fungoides and reticulum cell sarcoma (5-7). These observations agree with those of Van Seott and Winters (9) who showed that induction of hypersensitivity followed by challenge with nitrogen mustard or DNCB produces remissions of mycosis fungoides. Our data furthermore showed that administration of sensitizing agents selectively inhibited lesions of mycosis fungoides or reticulum cell sarcoma at concentrations of the sensitizing agent at which there is no evidence of a delayed-hypersensitivity reaction in the apparently uninvolved (normal) skin. Selective reactions of these lesions were also demonstrated in patients at the plaque or the ulcerated nodular stage of mycosis fungoides at concentrations at which the challenge reaction was essentially confined to the areas of neoplastic involvement.

Studies have been done on 14 patients (table 1). Of these patients, 10 had mycosis fungoides, and 4 had reticulum cell sarcomas. Of the patients with mycosis fungoides, 3 had generalized erythrodermic exfoliative disease. None of these 3 patients could be induced to develop immunity against DNCB, TEIB, phthallic acid, ortho and para phenylenediamine, or nitrogen mustard (HN<sub>2</sub>). One of these patients had a positive response to MV and to PPD; the other 2 patients were anergic and did not respond to the various cutaneous test antigens used. Challenge with PPD or MV failed to induce changes at the challenge site in the

TABLE 1.—Effect of immune challenge on lymphomas involving skin in patients

Tumors	Number of patients	Regressions
Mycosis fungoides		
Total erythrodermic stage	3	0
Eczematoid stage	2	2
Ulcerative nodular tumor stage	5	5
Reticulum cell sarcoma	4	4
	<hr/> 14	<hr/> 11

patient who had a delayed-hypersensitivity reaction to these preparations.

Of the 10 patients with mycosis fungoides, 5 had tumors at the ulceronodular stage of the disease. Two patients had the disease at the eczematoid stage. Delayed hypersensitivity to DNCB was induced in all 5 of these patients, although it took considerably longer (4-12 weeks) to elicit responses in these patients compared to normal individuals. Also the responses were relatively weak; *e.g.*, challenging doses of 1:10,000-1:100,000 were required, while in normal individuals or patients with epidermal neoplasms, responses were obtained at concentrations as low as 1 part per billion. Also, the responsiveness of patients with mycosis fungoides was apparently diminished in other respects. All 10 patients had delayed-hypersensitivity reactions to  $\geq 2$  microbial test antigens (*e.g.*, PPD, MV, vari-dase-V, P vaccine, CE, T, H, C, and B). All 10 patients showed a reaction to Schick antigen, thus indicating lack of antibodies against diphtheria toxin.

Because of the relatively long time required for inducing delayed hypersensitivity to DNCB and the diminished response to challenge with DNCB, the effects of microbial antigens to which the patients had retained immunologic memory were explored. Under identical investigative conditions, the response to intralesional administration of skin antigen was markedly more pronounced than the reaction in the uninvolved skin. Resolution of lesions injected with microbial antigens was observed after intralesionally induced delayed-hypersensitivity reactions (figs. 4-6).

Four patients with multiple reticulum cell sarcomas involving the skin were studied. One of these patients developed delayed hypersensitivity to nitrogen mustard but not to DNCB. The other



3 patients failed to develop hypersensitivity to several haptens including DNCB, TEIB, and  $\text{HN}_2$ , but responded to challenge with a number of microbial antigens (PPD—3, CE—3, H—1, MV—2, and varidase-V—2). Three of the four patients responded after immune challenge reactions by disappearance of challenged as well as untreated lesions. One patient, who showed a challenge response to MV and PPD, showed only partial or no regressions of treated lesions. Since this patient had lymph node and organ involvement and untreated lesions had failed to respond, she was subsequently treated with systemic chemotherapy.

Regressions in 1 patient lasted for an observation period of 6 months and appeared to be progressive while the patient remained under treatment with intralesional administration of CE. In this patient, most lesions including untreated lesions had grossly and microscopically undergone complete regressions. Initially, approximately 30 lesions were present; 5 residual lesions are still being given injections of CE at intervals of 3–7 days. Before initiation of the immunologic challenge regimen, the patient had rapid progression of disease, as indicated by an increasing number and enlargement of lesions. He ceased to develop new lesions after the immune challenge regimen was started, while approximately 25 lesions present at that time disappeared and 5 residual lesions were regressing.

The period of regression in another patient lasted 3 months and was terminated by death due to a myocardial infarction unrelated to the malignant disease. This patient remained in complete remission without immunologic challenge after he had cleared. The third patient had shown regression of all lesions for a period of approximately 6 weeks when she was placed on prophylactic chemotherapy, because of persistent hematologic abnormalities.

#### *Selective Effects of Immune Challenge in Metastatic Cancer*

Selective effects of local challenge reactions were demonstrated in patients with disseminated malignant diseases (Kaposi's hemorrhagic sarcoma, malignant melanoma, and metastatic adenocarcinoma of the breast) involving the skin and subcutaneous tissues (table 2). A study of 8 patients of a group of 14 in whom 11 sensitizing agents

TABLE 2.—Effect of immune challenge on metastatic malignant diseases in patients

	Number of patients	Regressions
Adenocarcinoma (breast)	5	5
Melanoma	6	2
Fibrosarcoma	3	0
Kaposi's hemorrhagic sarcoma	4	3
	<hr/> 18	<hr/> 10

were explored showed challenge reactions which produced more intense responses at tumor sites than in normal tissues.

In 3 patients with Kaposi's hemorrhagic sarcoma, tumors were eradicated after delayed-hypersensitivity reactions to DNCB (2 patients) and TEIB (1 patient). The patient who had responded to challenge reaction with TEIB subsequently had complete involution of all lesions and has remained free of active disease for an observation period of 6 years. It should be noted that "spontaneous" remission of Kaposi's hemorrhagic sarcoma has been observed and that definitive conclusions cannot be drawn from these observations.

In 4 of 6 immunocompetent patients with malignant melanoma who were studied in collaboration with Gerner (10), regressions ranging from partial to complete resolution of metastatic tumor were observed following delayed-hypersensitivity reactions to DNCB. In 2 of these patients, lesions exposed to immune challenge reactions apparently converted to benign "halo nevi" resembling benign moles as they "spontaneously" regressed. One of these patients, whose disease was rapidly progressing at the time of induction of hypersensitivity and subsequent challenge, remained free of disease for  $3\frac{1}{2}$  years. In the second patient, who also had rapidly progressive disease when the hypersensitivity regimen was initiated, observable lesions had converted to halo nevi or had completely regressed during the 6-month observation period. Regressions occurred in challenged as well as in unchallenged lesions, as determined by clinical observation and biopsies. The microscopic appearance of apparent halo nevi in these patients was consistent with that of the late-resolving stage of moles without evidence of residual malignant cells and with a dense mononuclear infiltrate at the site. Lesions of metastatic, malignant melanoma which had been biopsied before immune challenge were character-



ized by lack of a cellular infiltrate which was the basis for differentiating metastatic from primary malignant melanoma.

Five patients with metastatic adenocarcinoma of the breast were studied in collaboration with Dao, Rosner, and Milgrom (8). These patients had been exhaustively treated by standard and investigative methods and had failed or ceased to respond. Three of the patients had had bilateral adrenalectomy before immunologic challenge was initiated. In 3 patients, intense delayed-hypersensitivity reactions to a number of microbial test antigens were demonstrable. The other 2 patients with far advanced disease responded marginally to MV and CE but did not respond to other microbial antigens. Two patients developed delayed-hypersensitivity reaction to DNCB. The other 3 patients are being exposed to DNCB to determine whether delayed hypersensitivity to this agent can be induced over protracted periods.

The patients who reacted to DNCB have been under study for 6 and 9 months, respectively; they also reacted intensely to PPD, MV, and CE. One of these patients had 4 areas of involvement with metastatic disease: a large, fungating, ulcerated mass (10 × 10 cm in diameter) on the anterior chest wall (fig. 7), a small metastatic nodule (3 cm in diameter) overlying the xiphoid process (fig. 7), and a multinodular mass (approximately 10 × 5 cm) in the right adrenalectomy scar over an ellipsoid area with an exophytic component (2–3 cm above the skin) and a pleural effusion. The large metastatic lesion on the anterior chest wall was given daily injections of PPD, as was the mass in the adrenalectomy scar, while the third lesion was left untreated to determine whether distant effects would occur. After approximately 2 weeks of intralesional administration of PPD, the ulcerated area on the anterior chest wall became progressively smaller (fig. 8), as did the lesion in the adrenalectomy scar. A cream (20 µg PPD/g) was applied to the residual lesion on the chest wall, while intralesional administrations of PPD to the lesion in the flank were continued every 2 or 3 days. After approximately 3 months of daily topical administration of PPD, the ulcerated area on the chest wall had decreased to <2 cm in diameter. Repeated biopsies and exfoliative cytologic examination failed to show tumor. The denuded area was covered by granulation tissue, and re-epithelialization occurred (fig. 9). Evidently the malignant

cells were being removed while, at the same time, under the same conditions, and at the same site, normal tissues were able to grow and resurface at the site of previous tumor involvement. The lesion in the adrenalectomy scar decreased to approximately 5 × 3 cm and changed from a highly cellular mass to a densely fibrotic scar tissue which, however, on biopsy examination contained residual tumor.

The pleural effusion resolved within 3 months of PPD administration which, however, may have been a delayed effect of discontinuing estrogen therapy approximately 6 months earlier. The third lesion, which had been left untreated, decreased to approximately 2 × 1 cm in diameter.

The lesions continued to decrease in size, while PPD administration was discontinued for 3 and 4 weeks, respectively. There was no apparent recurrence of the tumor on the chest wall. The skin overlying the mass in the right flank at the site of the adrenalectomy scar underwent necrosis over an area approximately 2 × 2½ cm in diameter with ensuing healing. Although the tumor mass had continued to decrease during periods when immune challenge was withheld, PPD administrations were resumed for prophylactic purposes. During the 9-month period since PPD administrations were started in this patient, there was no evidence of new metastases.

The second patient with metastatic adenocarcinoma of the breast had multiple contiguous and isolated nodules infiltrating the chest wall at the site of the mastectomy scar and has been under study for 6 months. Intralesional administration of PPD resulted in necrosis of tumor nodules. Topical administration of PPD produced an intense reaction at the site of involvement but produced no detectable reaction in the uninvolved skin. Regression of lesions, as determined by biopsies, was negative for residual tumor at treated sites and is continuing while the patient remains under study. Topical PPD administration is being continued.

In a third patient with adenocarcinoma of the breast who had shown marginal immunocompetence, some response was observed after intralesional administration of combinations of MV and CE. Tumor nodules given injections underwent erythema and edema, markedly more intense than in the uninvolved skin. The patient had been under study for 5 months without showing clinically significant improvement, although objective changes

at the challenged tumor sites were apparent and microscopic examination had failed to reveal residual tumor in treated areas.

This patient had an intense reaction to Schick reagent in the normal skin. Schick reagent was subsequently administered to subcutaneous metastatic lesions, which underwent necrosis and subsequently healed with scar formation. Multiple biopsies of this area failed to reveal residual tumors for observation periods of 3 months.

Two other patients with metastatic adenocarcinoma of the breast reacted to PPD and marginally to MV and CE. Topical administration of PPD resulted in selective reactions at sites of ulcerated metastases and in regressions. Both patients are being continued on topical PPD administration and remain under study.

Three patients with fibrosarcoma who failed to develop hypersensitivity to DNCB, but showed responses to microbial test antigens, were challenged intralesionally with the agents (varidase-V, MV, and PPD) to which reactions in tumors were markedly more intense than in the normal skin. Necrosis of tumors occurred at sites of the reaction. These patients were in preterminal stages and could not be adequately followed.

The data obtained in these studies suggest that the principles of the effects of immunologic challenge can be extended to metastatic lesions of widely disseminated malignant disease. Antitumor effects vary considerably, from lack of response to complete involution of malignant lesions in different patients and at times in the same patient. The variations in tumor response indicate the need for determining the mechanism which accounts for these differences in the effects of cell-mediated immune challenge reactions. The data further indicate the unusual phenomenon of converting a malignant tumor to an apparently benign lesion at a stage characterized by progressive and rapid involution.

### Effects of Local Challenge on Distant Tumors

Challenge of a single lesion in 4 patients with mycosis fungoides and 3 patients with reticulum cell sarcoma as well as in 6 patients with multiple epidermal tumors was associated with reactions at distant, unchallenged lesions. This was observed in patients with epidermal cancers who showed responses to challenge at tumor sites to low concen-

trations of sensitizing agents (DNCB at a concentration of 1 part per 100,000,000 and TEIB at a concentration of 1 part per billion). These reactions resembled challenge responses, but were markedly less intense than those observed at the challenged site. Tumor resolution was not observed at distant sites.

In patients with mycosis fungoides (fig. 10) and reticulum cell sarcoma (fig. 11), analogous reactions were manifested at distant sites of tumor involvement after local challenge of a single lesion. In these patients, lesions at various distances from the challenged tumor site underwent partial to complete involution (figs. 12, 13) after they had shown the equivalent of a delayed-hypersensitivity reaction.

These data indicate that a systemic factor may produce reactions at sites distant from the unchallenged area. These observations are similar to those made on patients with cutaneous tuberculosis (lupus vulgaris) who show a "flare" reaction at distant sites of tuberculosis involvement when challenged with PPD.

Induction of intense delayed-hypersensitivity reactions in lesions of mycosis fungoides by intralesional administration of microbial antigens (PPD and CE) resulted in reactions resembling delayed-hypersensitivity responses in untreated lesions in 4 of 7 patients. In these patients, reactions were followed by microscopically proved regressions of untreated tumors. Regressions occurred in 3 of 8 plaques in 1 patient and were complete (several hundred lesions) in 2 patients. Regressions lasted from 2-18 months. During intralesional administration of PPD, lymph nodes proved by biopsy to contain mycosis fungoides and neoplastic reticulum cells underwent involution. Regressions of distant untreated as well as treated lesions during treatment with PPD have lasted up to approximately 1 year in 1 patient. In the same patient, however, some lesions failed to respond to immune challenge and new lesions developed during the observation period.

In 3 of 4 patients with reticulum cell sarcoma, resolution of lesions distant from the challenged site occurred, as described above. In 1 patient, ancillary and inguinal lymph node involvement regressed which, on aspiration biopsies, showed intense accumulation of pyroninophilic cells. Of the 5 patients with mycosis fungoides in this group, who had progressive disease which failed to respond



to other forms of treatment when immunotherapeutic approaches were initiated, 2 have been free of manifestations of disease for up to 2 years. Two other patients have been kept under control with marked improvement and have been able to return to normal activities. One patient has improved moderately; she continues to develop recurrences at treated sites as well as new lesions which, however, respond to intensification of the immune challenge regimen.

Of the 4 patients with reticulum cell sarcoma in this group, 3 showed complete clearing associated with local regressions following cell-mediated immune challenge reactions. Two of these patients died of causes other than malignant disease in their 3d and 6th months, respectively. One patient developed extensive, deep-seated disease despite complete regression of cutaneous involvement after cell-mediated immune challenge reactions for a period of 6 months. The patient who had shown partial response is free of disease but is being maintained on chemotherapy.

### **Concurrent Challenge With Multiple Sensitizing Agents**

Concurrent challenge with  $\geq 2$  sensitizing agents increased the intensity of challenge reactions (fig. 14), as compared to the effects of single agents. It was also of interest that a challenge response could be demonstrated when 2 agents were used concurrently at the same site at concentrations at which either agent alone did not produce an apparent reaction. These observations were explored in neoplastic lesions that had not responded to challenge with a single sensitizing agent.

It appears that the concurrent use of multiple sensitizers results in an earlier onset of tumor resolution and that eradication of tumors may be feasible, but not so with a single sensitizing agent. These observations may be of further significance in patients whose challenge responses are of low intensity; by the use of several sensitizing agents, a more adequate response may be elicited.

Three patients with mycosis fungoides were studied. They had had local but not distant regression of lesions after intralesional administration of microbial antigens (PPD, MV, varidase-V, CE, and H). Combination of  $\geq 2$  of these antigens produced reactions in the uninvolved skin at concentrations below the threshold at which single

antigens induced a challenge response. The reaction to combinations of antigens at tumor sites was considerably more marked than that in the uninvolved skin. It was subsequently found that concurrent administration of multiple antigens produced more lasting regressions of a larger proportion of untreated lesions than when single antigens were administered. Clearing of lesions lasting 10 weeks occurred in 1 patient with mycosis fungoides after concurrent administration of multiple antigens (figs. 15–17). In the same patient, regression of lesions lasted 10 days when the individual antigens were administered into different lesions.

In the other 2 patients, partial regressions were induced with concurrent administration of  $\geq 2$  antigens to the same lesions; these regressions lasted longer, and the patients had proportionately fewer active lesions than when these antigens had been administered separately to different sites.

While the observation periods are too short and the number of patients studied too small to allow more than exploratory observation, the data indicate a common trend, in that challenge reactions based on immunologic memory resulted in regression of treated as well as untreated lesions, that responsiveness appeared to be related to the intensity of the cell-mediated challenge response, and that the intensity of that response could be increased by concurrent administration of  $\geq 2$  antigens.

### **Combination of Local Antimitotic Therapy and Immune Challenge**

Local administration of antimitotic agents, *e.g.*, 5-fluorouracil (5-FU), has been found not to interfere with the challenge response to DNCB or TEIB (4). Concentrations of topically administered 5-FU, exceeding the tissue level of this agent attainable by systemic administration by at least 3 orders of magnitude, enhanced rather than inhibited the response to challenge with DNCB or TEIB (fig. 13); although systemic administration of this agent at standard doses inhibits specific cell-mediated reactions, it would appear, therefore, that locally administered antimitotic agents either do not adversely affect mature mononuclear elements (lymphocytes and macrophages) or that these cells are replenished from central lymphoid organs.

At concentrations at which 5-FU alone or DNCB alone does not produce reactions in tumors or nor-



mal skin (control), a response can be demonstrated when both agents are administered concurrently to the same site (figs. 18, 19). Protracted use of 5-FU at the concentrations used ( $\leq 1\%$ ) did not produce reactions, while DNCB at low concentrations induced a reaction of low intensity which took up to 5 days to develop. Apparently the administration of 5-FU enhanced the effects of the sensitizing agent.

These observations were explored in 17 patients with intractable epidermal tumors by concurrent use of antimitotic agents and challenge responses, resulting in eradication of tumors which had failed to undergo resolution when the antimitotic agent or the sensitizing agent was used alone over protracted periods. These observations suggest that concurrent antimitotic chemotherapy and immunologic challenge may be developed as a clinically valuable approach to cancer therapy (figs. 20–22).

## DISCUSSION

The data presented review studies carried out in collaboration with other groups within our Institution and at other centers. As indicated by previous studies, cutaneous tumors involving the skin, whether they arise from the epidermis or from deep-seated tissues, provide investigative flexibility facilitating the observation of phenomena not readily recognized when they occur in deep-seated organs. Their accessibility permitted the demonstration of selective immune antitumor activity which could subsequently be extended as a general principle pertaining to a spectrum of tumors of various types. Immunotherapeutic approaches based on the same principles were, therefore, applied to metastatic lesions and disseminated malignant diseases.

The findings of a high 5-year cure rate after immunotherapy of primary epidermal tumors, the early recognition of clinically undetectable lesions, and the reduced incidence of new malignant lesions in cutaneous cancer syndromes characterized by persistent development of tumors, therefore, may be of significance for immunotherapeutic approaches to other types of cancer. Regressions induced in metastatic lesions of melanoma and adenocarcinoma of the breast as well as disseminated lesions of cutaneous lymphoma indicate the need for a systematic investigation to determine the mechanisms by which analogous approaches may be developed for the treatment of these and

other neoplastic diseases at earlier stages. Studies demonstrating antitumor effects on malignant lesions located on the skin and elsewhere in the body suggest the operation of mechanisms which may be explored for immunotherapeutic approaches to inaccessible lesions.

The demonstration that premalignant lesions are eradicated before their progression to the malignant state may be of significance in regard to the level of transformation of normal cells at which they develop detectable antigenic changes and can, therefore, be differentiated from normal cells and eliminated. Immunoprophylaxis may extend to cells at the premalignant stage at which morphologic or biochemical characteristics cannot be recognized as differing from those of normal cells by presently available methods, but which can be detected and controlled immunologically. Studies in animals indicate that nonspecific immune challenge permits the development of nonmalignant papillomas as a result of exposure to chemical carcinogens, but prevents development of malignant lesions as long as challenge is being maintained. Studies on immunoprophylaxis may, therefore, be warranted in syndromes associated with a high incidence of malignant disease. In multifocal malignant diseases, in which progression of the disease may result from *de novo* neoplasia rather than by multiplication of already present malignant cells, immunoprophylaxis may contribute to the control of active or established disease.

The data presented here should be viewed as exploratory and are primarily indicative of feasibility and avenues justifying further pursuit, rather than as therapeutic procedures. Since it appears that immunotherapeutic approaches pertain to a spectrum of neoplastic diseases in man, it may be worth while to explore effects of cell-mediated challenge reaction in a variety of tumors not yet investigated in this respect. Accessible malignant lesions in the genitourinary tract have been studied in collaboration with Murphy, Albert, and Merrin (11). Analogous investigative approaches may be feasible in a number of tumor types located in the head and neck region, the intestine, and other directly or indirectly accessible regions.

The results obtained so far in metastatic and disseminated lesions indicate that at least palliative effects may be obtained at stages at which other methods of treatment have been exhausted or have failed to produce a therapeutic response. Further-

more, significant toxicity or generalized anaphylactic reactions have not been found in >150 patients under study for periods of up to 10 years. On this basis, further studies on immunotherapeutic approaches to disseminated malignant diseases would appear to be medically and scientifically warranted.

Immunologic memory as a basis for inducing antitumor effects by immunologic challenge reactions may be of theoretical as well as practical significance. From a practical point of view, it permits an immunotherapeutic approach to patients whose immunocompetence has been impaired in respect to their ability to recognize new antigens. We have found that this impairment frequently occurs before memory for immunologic events prior to the onset of the malignant disease is lost. Since most antimicrobial test antigens are relatively nontoxic, the parenteral administrations of these agents are under exploration and appear to provide results analogous to those obtained on local or cutaneous administration.

Theoretical considerations pertaining to the antitumor effects of cell-mediated challenge reactions elicited by microbial antigens suggest that a final common path for the antitumor effects of cell-mediated immune reactions is present, since the antitumor effects are essentially similar; whether small organic chemical agents (haptens) or relatively large molecular weight microbial antigens are used, it appears that the hapten-carrier complexes utilize pathways similar to those by which discrete, large, antigenic molecules exert their effects, even though the initial steps in the process may differ.

The observations that  $\geq 2$  sensitizing agents used concurrently produce more intense antitumor effects than single agents also have theoretical as well as practical implications for immunotherapy approaches. Our observations indicate that the combined administration of  $\geq 2$  antigens produces delayed-hypersensitivity responses in normal tissues as well as at tumor sites at concentrations markedly below those at which single antigens elicit challenge reactions. A mutual adjuvant response between 2 unrelated antigens evidently enhances the manifestations of the reactions to these agents where none can be demonstrated otherwise. Possibly similar considerations pertain in regard to eliciting a response to an otherwise weak or inadequate tumor antigen. The presence of several antigens

may, therefore, initiate a response which includes a reaction against the tumor antigen. This may also be so when a single antigen induces a differentially intense reaction at a tumor site under conditions in which no reaction is evident in normal tissues. Since the normal tissue contains only the administered sensitizing agent, at a minimal concentration, it may resemble the situation in which single antigens fail to produce responses by themselves, but can elicit reactions when  $\geq 2$  antigens are present simultaneously. Similarly, the tumor antigen alone fails to elicit an immune response but, combined with another sensitizing agent, may provide adequate conditions for an immune reaction against both the sensitizing agent and the tumor antigen.

The concurrent administration of  $\geq 2$  sensitizing agents to tumor sites produces selectively intense reactions which destroy the tumor, as compared to the minimal reversible changes observed in normal tissues under the same investigative conditions. The retention of selectivity in the presence of multiple antigens for a tumor antigen is analogous to the increase in the reactivity when more antigens are added, either in the normal tissues or at tumor sites. These observations may be of significance in respect to the effects of complex antigenic systems of intact microorganisms, such as bacille Calmette-Guérin (BCG) which contain a multiplicity of antigenic components.

It is of further interest to note that the dose-response relationship indicates that the intensity of cell-mediated immune challenge reactions is increased as the concentrations of the sensitizing agents are increased, once the threshold for inducing a detectable reaction has been reached or exceeded.

One possible mechanism of the increased intensity of the challenge response in the presence of several antigens is the increased number of immunologically committed lymphocytes congregating at the challenge site. Since the onset of the cell-mediated challenge system appears to depend on a threshold level of committed lymphocytes undergoing a response, the presence of larger numbers of reactive mononuclear cells may result in exceeding an otherwise subliminal stimulus.

Our studies have been mainly concerned with the antitumor effects of the cell-mediated system. Challenge reactions induced by Schick antigen, however, may be of interest, since a delayed com-



ponent of the reaction appeared to be ineffective, as indicated by simultaneous administration of toxoid, while the reactions without concurrent administration of diphtheria toxoid destroyed lesions of mycosis fungoides, reticulum cell sarcoma, and carcinoma of the breast. These observations suggest that humoral antibodies are a factor in producing cytotoxic effects under appropriate conditions, although as yet selectivity for tumor cells has not been established.

Preliminary studies on the inhibitory effects of blocking antibodies against the antitumor effects of cell-mediated challenge reactions have been explored in accessible lesions by the administration of antibodies against human  $\gamma$ -globulin to lesions of melanoma and mycosis fungoides which had failed to respond to cell-mediated challenge. The administration of antihuman  $\gamma$ -globulin alone to malignant lesions did not alter their course. When a cell-mediated challenge reaction—which, by itself, also did not affect the course of the neoplastic lesions—was superimposed, involution of the lesions followed. While the anti- $\gamma$ -globulin preparation may have removed blocking antibodies, the formation of a “sandwich” complex may have occurred with  $\gamma$ -globulins of the host interacting with components of the tumor cell surface (immunologically specific or high-affinity proteins), thus providing an antigen at the tumor cell surface in addition to the sensitizing agent applied topically. Sequence of events which followed would correspond to the development of a cell-mediated response to the products of the interaction. An immediate erythematous reaction supervened at the tumor site as well as in the normal control skin within approximately 15 minutes. This reaction progressed for the ensuing 24 hours, when the reaction faded in the normal skin, but continued at the tumor site for approximately 12 days. The superimposition of a cell-mediated challenge resulted in a more protracted reaction in the normal skin, which disappeared within 7 days, while it lasted for up to 20 days at the tumor sites. The implications of thus producing a specific tumor immunity by the use of  $\gamma$ -globulin-coated tumor cells are under investigation.

Combinations of chemotherapy and immunotherapy were explored previously (6). Initially, topical chemotherapy was superimposed on an immune challenge reaction. The intensity of the combined response considerably exceeded the re-

action to each modality alone without loss of selectivity for the tumor site. Systemic chemotherapy combined with local immunotherapy was subsequently explored. Agents used systemically included bleomycin and triacetyl azauridine administered at doses which were not immunosuppressive, as determined by skin tests. Indications of at least additive antitumor effects were obtained in patients with reticulum cell sarcoma and squamous cell carcinoma. Recently initiated studies used a pulsed regimen of high doses of methotrexate followed by citrovorum rescue as described by Djerassi (12); this regimen appears to permit the immune response of the patient to recover between successive chemotherapeutic treatments.

While the studies presented indicated the feasibility of immunotherapeutic approaches to several types of malignant disease, further pursuit of failures of this approach will provide meaningful information on obstacles to the more effective and wider application of immunotherapy. Intensive study is also indicated on the relatively small proportions of patients in whom, so far, immunotherapeutic approaches have been found to exert clinically significant antitumor effects. Further studies in this area may be of significance in tumor immunology, as well as provide information relevant to infectious diseases and other normal and pathologic processes in which immune mechanisms play a significant role. While the tumor system provides a challenging area for investigating complexities of delicately balanced immunologic systems, the converse is of major importance, since progress in the immunologic aspects of cell biology and the role of noncellular mediators and their regulatory mechanisms are of fundamental as well as applied importance.

Selective destruction of tumor cells in conjunction with the induction of a delayed-hypersensitivity challenge reaction at tumor sites appears as a complex phenomenon that may involve both cellular and humoral factors. Since tumor regression is observed in the absence of discernible effects on the surrounding normal tissues or the healing process, these factors must possess some degree of selectivity or specificity. Besides immunologically specific factors, nonspecific factors including the various noncellular mediators and cellular elements of delayed hypersensitivity may be operative. To what extent these cellular elements possess selectivity or specificity is only partially understood.



In addition to recognized immune phenomena, other discriminatory mechanisms may be significant.

The features of a delayed-hypersensitivity reaction include local accumulation of mononuclear cells in addition to increased capillary permeability and extravasation of plasma proteins. Both cellular and noncellular components of the delayed-hypersensitivity reaction can be expected to be involved in the observed destruction of tumor cells when the reaction is induced at the site of a tumor. The following factors seem involved: 1) The monocytes and the macrophages are the ultimate effectors of tumor cell destruction. 2) The selectivity for tumor cell destruction is mediated by antibodies cytophilic for the monocytes or the macrophages or by antibodies interacting with tumor-specific antigens on tumor cells, thus earmarking the tumor cells for interaction with the monocytes and the macrophages. Preliminary experimental observations in our laboratory support this view (14). Peritoneal macrophages from rats obtained after BCG sensitization and PPD stimulation will selectively destroy a number of different syngenic rat tumors *in vitro* (15) (figs. 23, 24). Serum components from tumor-bearing animals influence profoundly this *in vitro* interaction between macrophages and tumor cells (14). Thus we have evidence that macrophages can act as effector cells in tumor cell destruction and, furthermore, that serum factors (presumably antibodies) can influence the interaction between tumor cells and macrophages.

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FIGURE 1.—Patient with severe actinic damage of skin with long history of multiple premalignant keratoses, basal cell carcinoma, and squamous cell carcinomas. Multiple scars indicate frequent surgical procedures before immunotherapy.

FIGURE 2.—Patient responding to immunologic challenge regimen. Reacting areas are limited to sites of neoplasm without reaction in adjacent non-neoplastically involved tissues including scars from previous surgery unless they contain recurrence.

FIGURE 3.—Patient 6 weeks after therapy, showing complete recovery without additional scarring except for 2 residual areas responding after second course of immunotherapy. Initial course of immunotherapy completely eradicated numerous individual lesions.

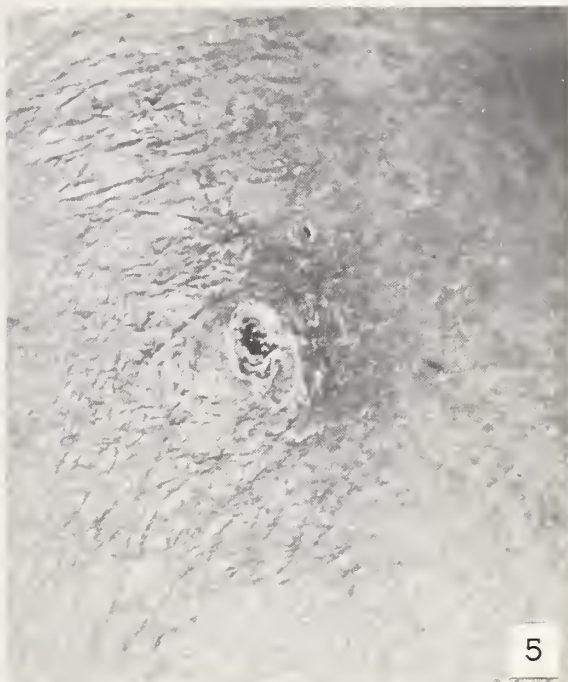


FIGURE 4.—Lesion of mycosis fungoides on upper arm prior to challenge with PPD.

FIGURE 5.—Response to PPD challenge in same lesion of mycosis fungoides as shown in figure 4.

FIGURE 6.—Site of previous involvement by lesion of mycosis fungoides shown in figures 4 and 5, three months after challenge with PPD. Postinflammatory pigment persists. Clinical and biopsy examination reveals the area to be free of mycosis fungoides involvement.



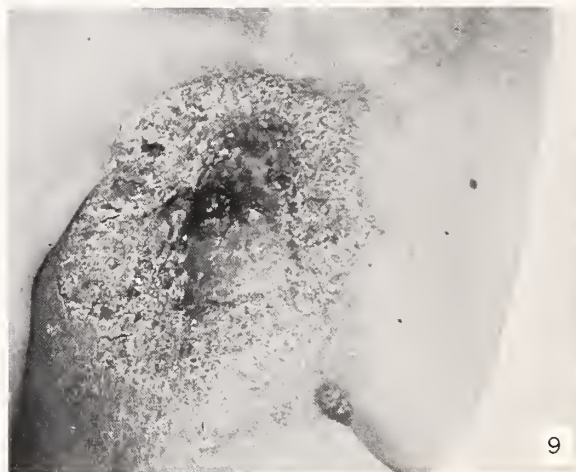
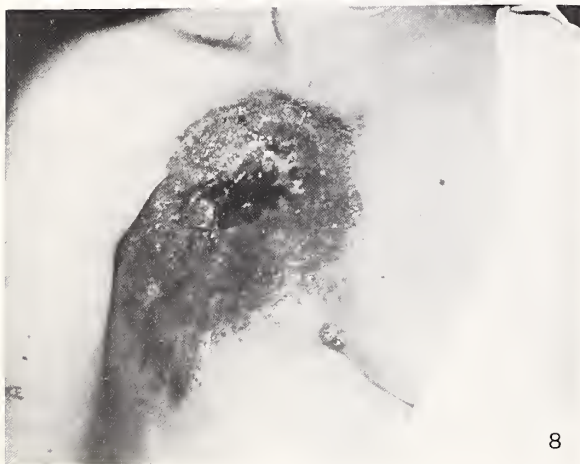
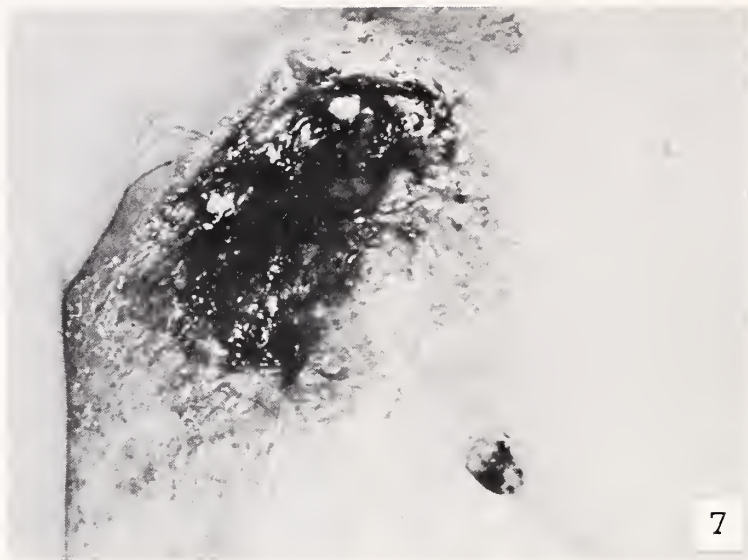


FIGURE 7.—Patient with ulcerative necrotic recurrent adenocarcinoma of breast following surgery and other therapeutic modalities before immunotherapy, showing intense response to administration of PPD at multiple sites within and at margin of lesion.

FIGURE 8.—Same patient as shown in figure 7 after course of multiple PPD administration intralesionally and perilesionally. Necrotizing tumor has involuted, permitting granulation tissue and re-epithelialization to proceed from the margin toward center of lesions. At this stage, PPD administration in topical form was initiated.

FIGURE 9.—Same area as shown in figures 7 and 8 after re-epithelialization. Repeated biopsies failed to reveal residual adenocarcinoma.



FIGURE 10.—Patient with widespread involvement of mycosis fungoides after challenge with PPD or DNCB at a single site (in this case lesion of right breast) which resulted in intense flair at distant unchallenged sites.

FIGURE 11.—Patient with reticulum cell sarcoma before immunologic challenge.



FIGURE 12.—Same patient as shown in figure 10 with extensive mycosis fungoides after reaction at sites distant from challenge, resulting in resolution of challenged as well as unchallenged lesions.

FIGURE 13.—Same patient as shown in figure 11 with reticulum cell sarcoma after challenge to single area and resolution of lesions at challenged as well as distant, unchallenged sites. *Square areas* to which challenge was applied revealed residual, postreactive pigmentation. Sites of previous involvement of unchallenged lesions also retain residual pigmentation free of tumor cells.





FIGURE 14.—Patient showing challenge reaction to a number of microbial antigens. Site at which PPD and MV was administered concurrently shows far more intensive reaction than sites at which individual antigens were administered alone.

FIGURE 15.—Patient with extensive involvement of mycosis fungoides who had partial responses after challenge reaction to single antigens.



FIGURE 16.—Same patient as shown in figure 15 after challenge with concurrent administration of intralesional H and topical DNCB. Reactions are marked with challenge sites as well as at distant, unchallenged sites.

FIGURE 17.—Same patient as shown in figures 15 and 16 after induction of challenge reaction to concurrent administration of DNCB and H. Challenged as well as unchallenged lesions which had failed to respond to challenge with single sensitizing agent underwent partial resolution.

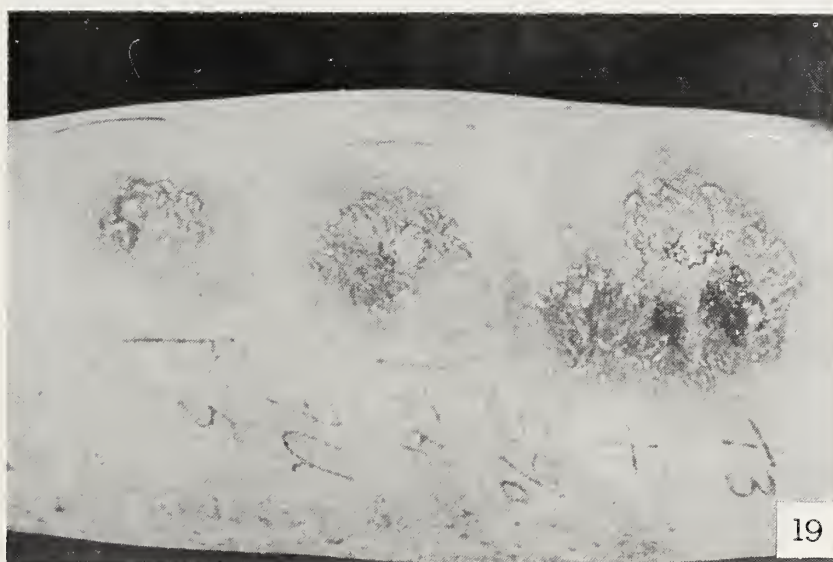


FIGURE 18.—Patient challenged by skin testing with several concentrations of DNCB ranging from 1 part per 1,000 to 1 part per million.

FIGURE 19.—Same patient as shown in figure 18, with markedly more intense reaction when topical 5-FU was applied concurrently with the same range of concentration of DNCB as shown in figure 18.





FIGURE 20.—Patient with arsenical dermatitis and multiple squamous cell carcinomas showing lesion of recurrent squamous cell carcinoma which had failed to respond to topical chemotherapy and immunologic challenge with DNCB.

FIGURE 21.—Same area as shown in figure 20 reacting to concurrent topical administration of 5-FU and challenge with DNCB (1:10,000). Note necrosis at site of involvement while surrounding skin underlying tissue is undergoing inflammatory reaction.



FIGURE 22.—Same patient as shown in figures 20 and 21 after resolution of tumor and reaction in adjacent tissue. Scars at previous site involved by tumor are due to multiple biopsy examinations which failed to reveal residual tumor.

FIGURE 23.—Tissue culture monolayer of a Fischer rat sarcoma induced by polyoma virus.



FIGURE 24.—Duplicate culture of sarcoma cells shown in figure 23, 48 hours after addition of Fischer rat peritoneal macrophages. Donor rat of macrophages had been sensitized by BCG vaccination and given intraperitoneal injection of PPD 48 hours before harvest of macrophages. *Note* extensive destruction of sarcoma cells.



## Immunologic Evaluation of the Cancer Patient During Immunotherapy<sup>1, 2</sup>

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**SUMMARY**—In an experimental immunotherapy program, in progress 5 years, 234 patients were given injections of either a crude homogenate of tumor tissue (HOM) or concentrated cell sap of tumor tissue (CCS). For purposes of comparison, our results from patients with disseminated malignant melanoma were: Of 34 patients with lesions that could be evaluated objectively and given injections of HOM, 7 showed a favorable clinical response. CCS injection gave a positive response in 3 of 10 patients. Ten percent of all patients receiving HOM had a delayed-hypersensitivity reaction at the site of injection compared to 62% receiving CCS. With insignificant progress thus far in the experimental immunotherapy programs, laboratory evaluation of these patients is crucial. Sera from 45 melanoma patients were available. In immunodiffusion tests, 3 of 31 sera from patients that received injections of HOM reacted to give a line of precipitation compared to 4 of 14 sera from patients that received CCS. Cross-reactivity with CCS of other tumors was limited, whereas all positive sera reacted with the CCS from 4 different melanoma patients. In immunoelectrophoretic studies, the reactive serum factor migrated like  $\gamma$ -globulin. When CCS was percolated through a Sephadex column, the reactivity resided in the first peak after the void volume; melanin was in the third peak. In complement-fixation studies, peak 1 of CCS was used with the immune sera of the 14 people given injections of CCS. Ten sera gave titers ranging from 10–320.—*Natl Cancer Inst Monogr* 35: 403–408, 1972.

TUMOR-SPECIFIC immunity in animals is a well-established fact (1, 2). The same phenomenon in man is a debatable issue. In 1965, Gold and Freedman (3) reported tumor-specific antigens in adenocarcinoma of the human colon. However, recently Reynoso *et al.* (4) described abnormal levels of carcinoembryonic antigen (CEA) in sera of

patients with cancer of sites other than the gastrointestinal tract. In 1968, Morton *et al.* (5) demonstrated antibodies against human malignant melanoma by immunofluorescence, and in 1970 Jehn *et al.* (6) described the preparation of a soluble antigen from malignant melanoma and its use for *in vitro* stimulation of lymphocytes. This report summarizes data on clinical material, because this material was presented elsewhere (7). Furthermore, to effect meaningful correlations, data are given on studies of cytotoxic, precipitating, and complement-fixing antibodies in the sera of malignant melanoma patients only. The subcellular fractionation and partial purification of the antigenic material will be described.

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## MATERIALS AND METHODS

*Patients.*—Only those with disseminated or locally incurable cancer were included in this experimental immunotherapy study. Preimmune serum samples were obtained. Group A (181 patients) was immunized intradermally and subcutaneously with 4–8 weekly injections of a 20% frozen-and-thawed homogenate (HOM) of tumor tissue (8). Group B (53 patients) was immunized with concentrated cell sap (CCS) and the same immunization protocol was used. With a few exceptions early in the study, patients were immunized with preparations from another patient having a similar tumor diagnosis. Within 3 days of the last tumor injection, an immune serum sample was obtained.

*Antigen preparation.*—Malignant melanoma tissue, obtained at surgery, was stored at  $-60^{\circ}\text{C}$ . A 20% mixture of diced tumor and cold extracting fluid, medium A (9), was homogenized in a Sorvall Omnimixer for 1 minute at full speed; the Omnimixer container was suspended in an ice-water bath. The nuclei and mitochondria were sedimented by successive centrifugations at  $1000 \times g$  and  $5000 \times g$ , both for 10 minutes. Microsomes were sedimented from the postmitochondrial supernatant at  $102,000 \times g$  for 75 minutes. The remaining supernatant was designated “cell sap” (CS). By use of an Amicon UM-10 membrane, CS was concentrated to one-tenth its volume. These subcellular fractions were prepared from: malignant melanoma tissue from 4 different patients, normal liver, and 7 other tumor types (*cf.* table 2).

*Serologic methods.*—Double-diffusion gel analysis was performed according to Ouchterlony (10). All experiments were done at room temperature, with 0.5% agarose in phosphate-buffered saline,  $p\text{H}$  7.2. Precipitation lines appeared after overnight incubation.

Immunoelectrophoresis was performed by the micromethod of Scheidegger (11) by use of Gelman equipment and barbital acetate buffer,  $p\text{H}$  8.6,  $\Gamma/2 = 0.05$ . Sera were subjected to electrophoresis for 1 hour at 300 v (constant voltage) at room temperature. Troughs were filled, and the slides were incubated overnight at room temperature in a humid atmosphere.

Complement fixation was performed according to Levine (12), with the following modifications: Reagent volumes were halved, to provide a total reaction volume of 3.5 ml. The antigen: antibody:

complement mixture was incubated at room temperature for 2 hours; sensitized sheep erythrocytes were then added, and the tubes were incubated in a  $37^{\circ}\text{C}$  water bath for an additional hour. The optical density of the supernatants was then determined in a spectrophotometer at 413 nm. Sera and antigen preparations were titrated for anticomplementary effect initially; appropriate controls were included in every test. Ten percent or greater complement fixation was considered significant.

*Chromatography.*—Gel-permeation chromatography was performed at  $8^{\circ}\text{C}$ , with Sephadex G-200 columns ( $105 \times 2.5$  cm) prepared as recommended by Pharmacia, Inc. Fisher-Porter chromatographic columns with minimal volume fittings were used. Medium A was the eluant. Either optical density at 280 nm was monitored with a Uviscan III connected to a Texas Instruments RectiRiter recorder, or the individual tubes were read in a Beckman DU spectrophotometer at 280 nm.

Protein concentrations were determined by the biuret method (13) with the use of  $E_{1\text{ cm}}^{1\%} = 2.8$ .

## RESULTS

Of 181 patients given HOM injections, 51 failed to complete the study, 34 were in the subjective group, and 96 could be evaluated objectively (measurable pulmonary lesion, skin or subcutaneous lesion). Twenty-two (23%) patients in the objective group responded clinically, and 10% had a delayed-hypersensitivity reaction (DHR) at the injection site (7). Of the 34 patients with disseminated malignant melanoma, in the objective group that were given HOM injections, (21%) responded favorably.

Of the 53 patients receiving CCS injections, 1 failed to complete the program, 17 are still under treatment, 14 were in the subjective group, and 21 were in the objective group. Since 4 of these patients also received concomitant intra-arterial drug therapy, only 17 were available for assessment of immunotherapy alone; 4 of these patients showed objective evidence of response. Interestingly, 62% had a DHR at the injection site. Of the 10 patients with malignant melanoma in the objective group, 3 (30%) had a positive response. In the entire series of 234 cases, there was no evidence of enhancement of tumor growth and no tumors developed at the site of injection.

Initial gel-diffusion studies, with the use of pa-

TABLE 1.—Reactions of patients' immune sera against malignant melanoma CCS

	Group A	Group B
Number positive in ID	3/31 (9.7%)	4/14 (28.6%)

tients' sera and all 4 subcellular fractions, indicated that only CCS was reactive, giving a line of precipitation in immunodiffusion (ID). The results are summarized in table 1. A total of 3 of 31 sera (9.7%) from group A patients and 4 of 14 sera (28.6%) from group B patients reacted with CCS. Melanomas from 4 different patients were used to prepare CCS; all 4 CCS preparations were reactive.

The positive ID results obtained with 4 different melanoma CCS preparations led us to test the specificity of the positive reactions with regard to CCS prepared from 7 other tumors of different histologic type (table 2). In group A, 2 of the 3 positive sera cross-reacted only with osteogenic sarcoma CCS. In group B, 1 patient's serum cross-reacted with 5 of the 7 CCS preparations. The other 3 sera did not cross-react. Thus, except for 1 serum from group B, there was a restricted specificity for CCS from melanoma. A CCS preparation from a surgical specimen of normal human liver (trauma) was tested against the sera from 7 patients. Undiluted liver CCS and serial dilutions to 1:16 were negative, indicating that the reactivity observed with tumor was not shared with normal liver.

Immunoelectrophoresis was performed to determine the nature of the reactive serum factor. Electrophoresis of an immune serum sample from each positive patient was followed by addition of mela-

TABLE 2.—Positive immune sera of melanoma patients tested by immunodiffusion against tumors of different histologic types

CCS prepared from*	Positive/tested	
	Group A	Group B
Melanoma (7.4)	3/3	4/4
Cancer of ovary (4.0)	0/3	1/4
Cancer of colon (6.5)	0/3	0/4
Renal cell cancer (1.4)	0/3	1/4
Cancer of lung (3.3)	0/3	1/4
Liposarcoma (5.5)	0/3	0/4
Osteogenic sarcoma (5.4)	2/3	1/4
Paraganglioma (1.5)	0/3	1/4
Normal liver (8.9) undiluted and diluted 1:2, 1:4, 1:8, 1:16	0/3	0/4

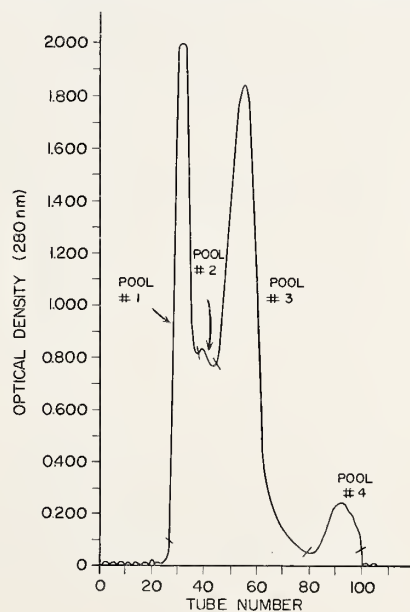
\* % Protein for each CCS appears in parentheses.

TABLE 3.—Distribution of titers in complement-fixation studies of sera from melanoma patients

Titer	Number of patients
10	1
20	1
40	2
80	2
160	1
320	2
640	0
1280	0
2560	1

noma CCS to each of the troughs. A single arc of precipitation developed in each case (fig. 1); comparison with a pattern from control normal human serum indicated that the reactive serum factor migrated as a  $\gamma$ -globulin.

Gel-filtration experiments were performed to partially purify the reactive antigen and to provide a suitable antigen for complement-fixation tests. Percolation of melanoma CCS through a Sephadex G-200 column resulted in the elution of 4 peaks (text-fig. 1). Peak 1 eluted immediately after the void volume, and scattered light after concentration by ultrafiltration. This peak accounted for about 33% of the eluted material. Peak 2, judging from the elution pattern, was a combination of the preceding peak 1 and the succeeding large peak 3; it accounted for about 17% of the eluted material.



TEXT-FIGURE 1.—Elution pattern of malignant melanoma CCS applied to a Sephadex G-200 column.



Peak 3 (42% of the eluted material) contained the melanin from the tumor. Peak 4 (7% of the eluted material) was water clear and eluted near the end of the experiment. Each peak was reduced by ultrafiltration to the volume of the originally applied sample. The concentrated peaks were tested immunoelectrophoretically against patients' sera. Only peak 1 reacted with these sera to give an arc of precipitation, indicating the presence of the reactive material in peak 1 only.

Complement-fixation studies were performed with the immune sera of the 14 people in group B. When serial dilutions of serum were made, 10 of 14 sera gave titers ranging from 10–320. The distribution of the 10 patients' serum titers (table 3) ranged from 10–2560, with most titers falling between 40 and 320. Thus, the serum factor in these patients' sera could react with the antigenic material in peak 1 to fix complement.

## DISCUSSION

Since clinical immunotherapy programs are still in experimental stages, methods of evaluation or specific types of response were not presented. These can be scrutinized in (7). Ironically, little has been gained in clinical responsiveness in the several programs. Hence laboratory evaluation of the cancer patient, as carried on by the Hellströms (2), Gold and Freedman (3), and Morton *et al.* (5), is crucial for progress in immunotherapy.

In the 234 patients given injections of different cancer preparations (HOM or CCS), no evidence of enhancement of tumor growth or tumor takes was noted. While the increase of clinical responses seen in those melanoma patients receiving CCS compared to HOM (30 vs. 21%) might seem noteworthy, emphasis should not be placed on these figures due to the heterogeneity of the groups. Furthermore, the greatly increased incidence of DHR at the site of CCS injections compared to HOM injection sites is suggestive but may not be a reaction to tumor antigens.

In our reports of preliminary studies (8, 14), serologic response of the patient was evaluated primarily by primary-culture inhibition test (15). This test is now used to evaluate each patient's response but, instead of a visual interpretation for cytotoxicity, isotope release ( $^{51}\text{Cr}$ ) is used.

However, when some of the sera from melanoma patients were found to react in immunodiffusion

tests with CCS only, more classical serologic tests were available.

Our initial serum analysis consisted of gel double diffusion with the homologous antigen. This enabled us to screen available sera from 45 melanoma patients to compare and contrast groups A and B. We found that the patients in group B showed a higher percentage of reactivity than those in group A. Since group B was immunized with CCS, rather than HOM, one might expect it to show a higher reactivity. The interesting point which arises in table 2 is that there was a limited cross-reactivity with other tumor types. Immunization with either HOM or CCS did not lead to a broad spectrum of reactivity with other tumors or with a CCS preparation of normal liver, and the cross-reactivity which did occur cannot be explained by blood-group incompatibilities alone. Thus, it seems an antigen is present in malignant melanoma and is detected by the sera of these patients, and that this antigen is not detected in CCS from other tumor types or in normal liver CCS. Jehn *et al.* (6) reported the presence of a soluble antigen, extracted from malignant melanoma tissue, which could stimulate autologous lymphocytes. This antigen, like ours, was not associated with the melanin of the tumor. However, it was said to have a molecular weight  $<40,000$ . In contrast, our antigenic material must have a minimum molecular weight of 800,000, since it eluted immediately after the void volume when percolated through a Sephadex G-200 column, indicating total exclusion from the gel. However, these differences may be more an artifact of preparation than real, reflecting differences in the initial homogenization of the tissue.

Immunoelectrophoresis experiments were performed to identify the serum protein reacting with the CCS. By electrophoretic mobility, the serum factor was a  $\gamma$ -globulin, corresponding closely to the  $\gamma$ -globulin of the normal human serum control. This agrees with the work of Morton and co-workers (5, 16) who demonstrated that the reactive factor in both immunofluorescence and microcytotoxicity tests is a  $\gamma$ -globulin, prepared by ammonium sulfate precipitation.

We next tried another serologic test, unrelated to precipitation, to further support the concept that the serum factor was an antibody. We used the quantitative micro-complement-fixation test. It was necessary to partially purify the CCS preparation by gel-permeation chromatography be-

cause CCS from malignant melanoma is a highly viscous, melanin-containing preparation, unsuitable for use in a photometric procedure. Table 2 lists the protein concentrations of the various CCS preparations; these ranged from 1.5–8.9%. With this fractionation technique, 67% of the extraneous material, including the melanin, was removed, giving a preparation, containing the antigen(s), that was suitable for complement-fixation studies. Sera from 6 of 10 patients (60%) had a significant titer of complement-fixing antibody, demonstrating that the antibody in the serum could fix complement in the presence of homologous antigen. This percentage is lower than that reported by Eilber and Morton (17) for complement-fixing antibody against human sarcoma; however, it is significantly higher than their values reported for cross-reactivity, the highest of which was 20% for blood-bank donor serum. The titers of our positive sera were within the same range as those reported by them for sarcoma.

Thus, it can be seen from our work and the work of others that man can respond immunologically to autologous and homologous tumor. While data herein reported describe only skin reactivity and humoral antibody determinations, this should not be construed as lack of appreciation of cellular immune factors. However, results of methods used for evaluation of cellular immunity, other than the test developed by the Hellströms (2), are so preliminary that they cannot be included. Studies on migration-inhibition factor and lymphotoxin are in progress. Finally, while the patient with a meta-

static lesion that can be evaluated objectively can be used appropriately as his own control, most patients at this stage have so much tumor that one would not expect active immunization to be sufficient to effect a response. Therefore, those patients with small amounts of tumor that cannot be evaluated objectively as well as those with very large amounts of tumor, requiring perhaps radiation therapy or chemotherapy with immunotherapy, must be studied by prospective match-paired programs. For the last year we have been conducting several such programs (table 4). Immunologic evaluation of the patients' response to the modalities used in these programs remains the real hope for progress.

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TABLE 4.—Prospective match-paired programs at Kansas University Medical Center

Program	Match-pair for:*	
	Control group	Treatment group
1) Ovarian cancer	Alkeran—5th wk	Alkeran—5th wk
2) Colon cancer	5-FU†—weekly	CCS other wk
3) Lung cancer	<sup>60</sup> Co—wk 1–3, 6–8	5-FU and CCS weekly
4) Melanoma, sarcoma	CCS—8 wk	<sup>60</sup> Co—wk 1–3, 6–8, CCS—wk 4–6, 8–10
		CCS—8 wk, transfer factor/wk × 12

\* All patients are incurable and studied initially before each modality and at conclusion for DHR and humoral and cellular immunity.

† 5-FU = 5-fluorouracil.

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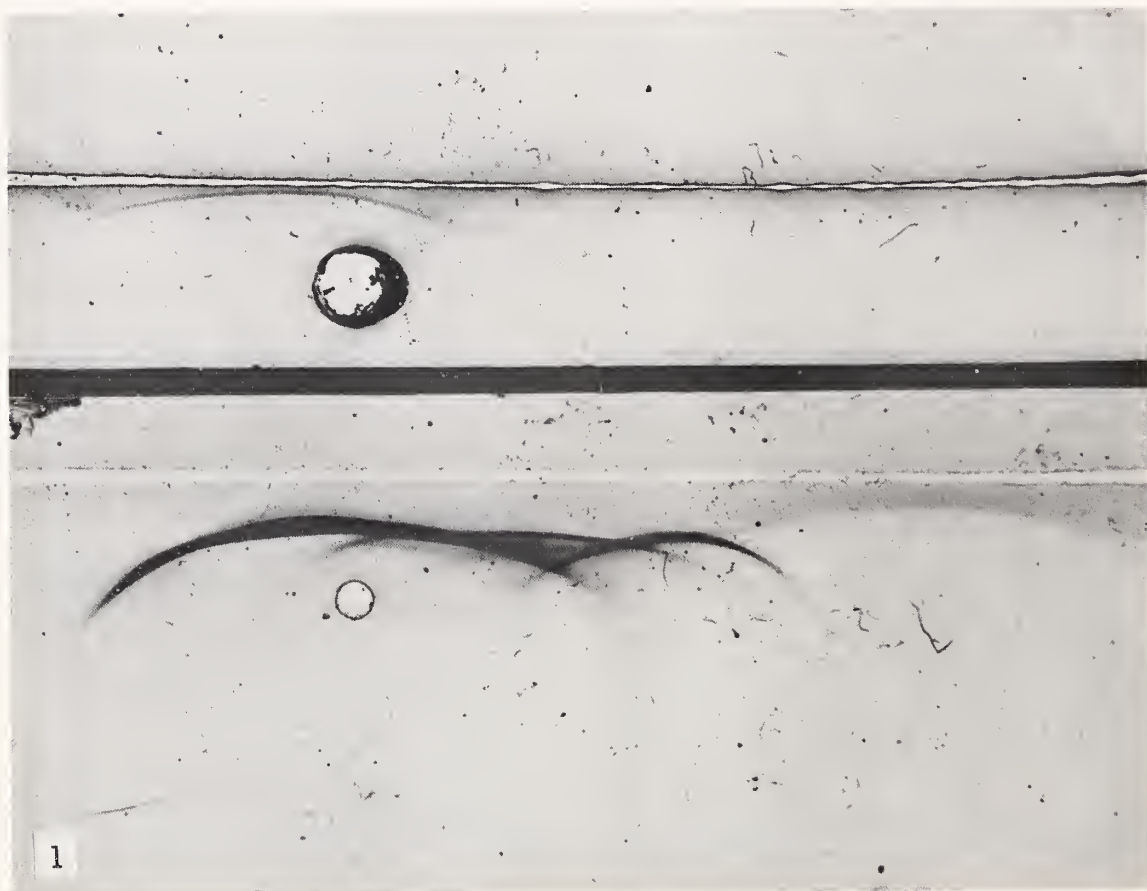


FIGURE 1.—Immunoelectrophoresis pattern of patient's serum (*top well*) against malignant melanoma CCS in trough compared with normal human serum (*bottom well*) developed with a goat anti-normal human serum antiserum. Cathode is to *left*.



## DISCUSSION

**E. Klein:** I would like to exercise the prerogative of the chair and ask the following questions from the audience before we launch into discussion. Has anybody had patients treated with immunotherapy, and what were your results?

**J. W. Thomas:** Bacille Calmette-Guérin (BCG) was used to prevent recurrence in 4 patients with extensive reticulum cell sarcoma and undifferentiated lymphoma. Standard irradiation and chemotherapy were used and, when patients were clinically free of disease, BCG vaccination was started.

The scratch procedure to the 4 extremities was used as per Dr. Mathé—every 4–6 weeks. All patients are alive and, at present, clinically disease-free.

Patient	Diagnosis	Recurrence	Duration of immune therapy (months)
1	Reticulum cell sarcoma	×2—treated with irradiation	30
2	Undifferentiated	None	20
3	Reticulum cell sarcoma	None	12
4	Undifferentiated	None	8

These results suggest that BCG given in these patients did not lead to enhancement, and a matched clinical trial is currently underway.

We had used oral BCG in a patient with multiple metastases to her small bowel. The patient had an entero-anastomosis for obstruction which gave good results, but she returned 6 weeks later with recurrence of abdominal distress and increasing weight loss. She was vaccinated with vaccinia and intracutaneous BCG, and she was given one blood transfusion from another patient with melanoma who had been immunized to vaccinia and BCG and who was receiving vaccinia and purified protein derivative (PPD) therapy for subcutaneous metastases. After the transfusion, subcutaneous vaccine was given for 4 weeks, and increasing doses of oral BCG (Connaught) were given twice weekly. Eight weeks after oral administration of BCG was begun, at 120 mg of BCG per week, symptoms of partial obstruction appeared. Currently the patient is being treated with 40 mg BCG orally each week which results in pain in the tumor masses 24–48 hours after BCG. The main tumor mass has been reduced to 50% of the original size. The patient gained 11 pounds. We really think that this is a route that might be considered further.

**J. Stjernswärd:** We have a series<sup>1</sup> where the new point is that dinitrochlorobenzene (DNCB)-induced delayed-hypersensitivity reaction will result in regression, not only

of primary skin cancer, but also of solid tumors, like carcinoma of the breast metastatic in the skin, mycosis fungoides, and various lymphomas in the skin. Of 21 small tumors, 13 regressed. Usually they did not disappear after a single application of DNCB, but we had to be rather aggressive, reapplying it many times, especially for a complete disappearance of mammary carcinoma nodules.

But a clear difference exists, which I think would be an important point to state more firmly in Dr. Klein's series and to analyze if really a true phenomenon exists. You reported that there is regression of distant tumors unchallenged by the allergen when you challenge one node. The results would thus indicate a more general systematic effect. This we have no experience of in our series which is very little though. But this observation may be important for elucidating the mechanisms behind observed regression more specifically. From most of our experimental data obtained from analysis of the mechanisms, close contact is postulated to be necessary.<sup>2</sup>

I would also like to point to the earlier data of the Kleins who show that, if you mix a few syngenic tumor cells with allogenic tumor cells which will be rejected, the syngenic cells will still not be rejected. This would indicate a certain specificity of the rejection mechanism(s).

Another point is the data that Dr. Zbar demonstrated; *i.e.*, if you mixed tumor A and B and have sensitized against one of them, you will have a nonspecific regression or delay in the outgrowth of the other tumor component, though the host has not been specifically immunized against the latter.

This could mean that nonspecific factors like lympho-stimulating factors, factors released increasing vascularization, or factors committing unspecific bystander cells, etc., could be utilized.

To conclude, it would be very important to elucidate what mechanisms are responsible for the observed results. Is it a specific effect, or a combination with an unspecific effect mediating a lot of factors? We could then be able to utilize the findings for therapeutic manipulations in a more general sense, *e.g.*, against minimal tumor cell doses in the body as such. But, to start, is the effect localized only as we think or is it general as has been claimed today?

**A. LoBuglio:** We ought to also address ourselves to utilization of materials like transfer factor as a means of immunotherapy, and this is presently our working area. We have studied a number of people, and we are identifying immune donors of transfer factor, using migration-inhibition factor (MIF)-released antigen. We have, for example, one patient with alveolar sarcoma who was unable to make MIF, though he was able to undergo lymphoblastic transformation.

He had an identical twin who was immune by both assays and who, when the transfer factor was developed

<sup>1</sup> STJERNSWÄRD J, LEVIN A: Delayed hypersensitivity-induced regression of human neoplasms. *Cancer* 28:628 640, 1971.

<sup>2</sup> UHR JW, *et al*: Immunological Intervention. New York, Academic Press Inc., 1972.

from his healthy twin, could produce a striking increase in MIF release in the patient. This did not result in clinical remission although he had extensive disease.

**Klein:** Thank you very much. Dr. Levy.

**N. Levy:** I would like to present another facet of this problem. Although I am not personally involved in immunotherapy, I have had the opportunity of studying 1 patient with melanoma. I used the microcytotoxicity test of the Hellströms. I would like to present some data on a temporal association between BCG immunotherapy, the appearance of a blocking factor, and the rapid clinical demise of the patient, all of which can be studied by the microcytotoxicity test.

This male patient in 1968 had had a solitary melanoma lesion on the upper extremity. This lesion was excised, and a radical axillary node dissection was performed. The axilla at that time was said to be free of tumor. Two years later, he developed a solitary intracerebral lesion, and this is when he came into my study. We studied him 3 times in the following 9 months. At each of these instances, he showed activity of cell-mediated immunity as measured by microcytotoxicity (e.g., between 60 and 80%) with no evidence of a serum-blocking factor. In June, 1971, he developed a small lesion of the chest wall and a small lesion of the tongue; each lesion was 1 cm in diameter. These lesions were not excised; instead, BCG immunotherapy was initiated in the chest-wall lesion. I studied the patient 2 days before BCG immunotherapy; at that time, again, he had good cell-mediated immunity and no evidence of a serum-blocking factor. Then, 2½ weeks later, after BCG injection, he developed multiple metastatic disease. The lesions on the chest wall and the tongue had grown to about 4 cm in diameter. Various radiologic studies revealed that he had widely disseminated disease. I'm not trying to make a cause-and-effect relationship. All I'm trying to point out is that no one here using BCG immunotherapy has the *in vitro* assay procedures to quantitate the appearance of blocking factor.

**Klein:** Dr. Levy, since you come from Duke University, could you tell us something about the data from the study of Dr. Ziegler?

**Levy:** I'm really unable to do that.

**D. M. Mumford:** We are involved in 2 programs. One series is at the M. D. Anderson Hospital with children, in which we sought to duplicate Dr. Mathé's work on immunotherapy. We have also studied some of the childhood melanomas, using irradiated tumor cells and/or diphtheria-parvum, DNCB.

At the International Cancer Conference, I reported on a second series which has been studied for the past 5 years. We have approximately 30 patients in this group in which we have given the irradiated tumor cells and/or adjuvants, including BCG, diphtheria-parvum, DNCB.

We have also done intranodular injections with some tumors, as Dr. Morton has carried out. We have seen similar types of responses in terms of local regression of the injected nodules and regression of noninjected nodules.

We are currently analyzing the data on survival. In the stage group that we used, which are patients with disseminated disease, most of whom have had chemotherapy, radiation, surgery—everything under the sun—we have

done immune profiles *in vitro*, including colony-inhibition, microcytotoxicity, lymphocyte transformation, MIF, etc. All these patients are not very good immunologic responders. Nonetheless, in this group of patients, although statistically they should have had a 1-year survival rate of 10 or 15%, they have had a 1-year survival rate of 50–60%. We are now calculating our 5-year survival rates.

**J. Gutterman:** Currently in the Developmental Therapeutics Department at M. D. Anderson Hospital, in collaboration with Dr. E. Hersh, Dr. G. Mavligit, and Dr. C. McBride, we have begun a program of immunotherapy in melanoma and have just begun a program of immunotherapy with BCG and leukemia cells in acute leukemia.

In our melanoma study, we are using an immunoprophylactic program and are studying different BCG strains; in effect, both the Tice Chicago strain BCG which is a lyophilized preparation and the fresh Pasteur strain from Paris. Trials in man with melanoma and also some animal experiments have suggested that different strains of BCG may have quite varied effects on the immune response.

We also are looking at different schedules (weekly scarification or once-a-month scarification) and studying various dose levels of the BCG. We are using high doses, about  $6 \times 10^8$  organisms, versus low doses, down to  $6 \times 10^6$  organisms.

With this type of approach, we hope to answer some of the questions that really have not been approached in immunotherapy trials in man: What is the optimal schedule? What is the optimal dose? Is one strain superior to another?

Although it is a little too early for us to report on definitive clinical results, I would like to mention one very important finding. We have had 4 patients, 2 with malignant melanoma, 1 with acute leukemia, and 1 with reticulum cell sarcoma, who were completely anergic to a battery of skin test antigens before the use of BCG. These are the type of patients as a group who, as Dr. Morton has clearly shown, have a very poor prognosis. All 4 patients have been on weekly BCG for 2–6 months now, and none of them has had a relapse. But the most interesting thing is that all 4 patients have converted at least 3 of the skin tests to positive. Hopefully this nonspecific increase of the general immunocompetence will be associated with an improved clinical prognosis.

**Klein:** Dr. Gutterman, do you have data on patients?

**Gutterman:** I cannot report any clear-cut data yet.

**Klein:** Thank you very much. Dr. Kennedy.

**J. E. Kennedy:** We have just begun to use immunotherapy on melanoma patients who have developed multiple metastases in spite of various combinations of surgery, radiotherapy, and chemotherapy. Three out of three patients with known metastases at the time immunotherapy was initiated showed clinical evidence of a specific attack on their metastases 36–48 hours after BCG- and neuraminidase-treated melanoma cells had been injected subcutaneously at a distant site. Subcutaneous nodules and the site of rib metastases became painful; a patient with a brain secondary nodule developed nausea and vomiting for 24 hours and a severe headache for 6 days. To avoid the excessive skin breakdown caused by large doses of BCG given subcutaneously, we have begun to use BCG by mouth, 22 mg every 4 days, for maintenance.



**Klein:** Does anybody have data on neuraminidase, or otherwise enzymatically modified tumor cell antigens? I think the only thing that we ought to refer to is the study by Dr. Ziegler. Apparently there is an improved antitumor effect if cells that have been treated with neuraminidase are used for vaccination. I would also like to briefly refer to the data of Dr. Sokal. His study is analogous to that of Dr. Mathé. He had a group of 15 patients with chronic myeloid leukemia who received conventional therapy. Half of them at clinically equivalent stages also received BCG, with or without allogenic tumors. The vaccinated group exceeded the life expectancy, and there is about 100% improvement in survival as compared to patients treated by conventional methods, as contrasted to those who also received added immunotherapy. This is of enormous significance.

**S. L. Leikin:** I would like to call attention to the fact that both the British Concord study and the children's cancer Group A study, using induction with chemotherapy and then BCG, have not supported Dr. Mathé's results.

**G. Mavligit:** I would like to comment on Dr. Ziegler's study. There is one major defect in that study, at least in my opinion, regardless of the small number of patients: the absence of the natural history of these patients before they were put on BCG. It is our experience that, if a patient has been free of disease for 1 or 2 years, his chances of not developing disease and of not having a relapse are much better than a patient who had a recent or increased incidence or recurrent cysts before he was put on BCG. This information is missing from Dr. Ziegler's study.

**Klein:** We will be discussing these comments later.

**Guterman:** I want to make a comment on the previous statement that the Concord study and the work from leukemia Group A failed to confirm previous immunotherapy trials in leukemia. In all these trials, there have been differences in the amount of BCG given, the route of administration, as well as the strain of BCG used (Glaxo, Tice, Pasteur). In addition, the amount of chemotherapy has varied from trial to trial. Therefore, I think a great deal of very careful work taking into account all these variables needs to be done before we are clear as to the exact role of immunotherapy in leukemia and other malignancies. Again, I would like to emphasize that it is very important to state clearly these variables in comparing one clinical trial with another.

**H. T. Wepsic:** I know of 3 patients who have been treated in Los Angeles for carcinoma *in situ* of the vulva, with Dr. Klein's technique of applying DNCB. All 3 of these patients have responded very well.

**Klein:** If there are no more comments on patients, are there any comments on different categories of disease that have been approached immunotherapeutically? Are there any data on prevention? Does anybody use specific or non-specific immunity on prevention? Or are there any suggestions? Are there any data or comments on the Montreal study with BCG? Dr. Rosenthal.

**S. R. Rosenthal:** There is unanimity of opinion that the immune mechanism, cellular or humoral, is operational in the control of neoplastic progression. It is also established that neoplasia is going on continually in the human host. Much has been discussed on the treatment of established

tumors and leukemias in which stimulation of the immune system has been successful in bringing about prolonged remissions. I wish to emphasize that prophylactic vaccination to stimulate the immune system so as to limit neoplasia before clinical disease is manifest should be given high priority. Experimentally there is good evidence that vaccination with BCG can inhibit the growth of transplantable tumors. Suggestive clinical evidence indicates the mortality from leukemia is one-half as great in children vaccinated with BCG at birth as compared to children not vaccinated. It is also known that cancer patients who react to tuberculin or to sensitizing antigens have a better prognosis than cancer patients who do not react to these antigens.

It is proper that prophylactic vaccination with BCG should be considered, since the vaccine is known to be safe and harmless (one-half billion vaccinated throughout the world against tuberculosis), and this vaccine is known to be one of the most potent stimulators of the immune system.

**Klein:** I have no further specific points to make, and the floor is open for a general discussion.

**A. J. Girardi:** Dr. Humphrey, in preparing your vaccines, why did you select the methods that you used when these methods are known to destroy tumor-resistant antigens in model systems where the antigens are known to exist?

**L. J. Humphrey:** The methods were worked out perhaps 1½ years before we started our clinical study, and by this method the particular postribosomal fraction reacted with immune serum and gave sufficient data for us to initiate a clinical study.

**B. Zbar:** I have another question with respect to the antigen you used for immunizing patients. What evidence do you have that the antibodies you detected were indeed reacting with tumor antigens rather than with histocompatibility antigens or any other antigens you might see in an allogenic situation?

**Humphrey:** We are now carrying out a prospective study with Dr. Jones in which the sera are analyzed for histocompatibility antibodies. So far no pattern with respect to blood-group reactivity has been established in different patients. I think you raised a good point, though, which is that this is supposedly a sequestered antigen, and one that may not be expressed at the cell surface. We don't have any evidence of this antigen reacting *in vitro*. This may be a spectator antibody.

**Levy:** I would like to finish that presentation I began a few minutes ago. This patient was studied again 3 weeks after BCG immunotherapy. He had cell-mediated immunity (as measured by the microcytotoxicity test) of about 70%, and he had a serum-blocking factor that was able to abrogate 100% of the cell-mediated activity at a 1:4 dilution. These sera were diluted, and they went out to about 1:15. The patient died with widespread melanoma 22 days after BCG immunotherapy.

**D. L. Morton:** I would like to comment that malignant melanoma, by its natural history, is a very variable disease. While we were waiting for the results of DNCB skin tests, we have seen patients go from 1 or 2 nodules to hundreds of nodules and die within 2 weeks. Even though blocking antibodies appear within a period of 2-3 weeks following BCG, it doesn't mean that enhancement has occurred due



to the BCG because this can occur in the natural course of the disease.

**W. D. Terry:** I just wanted to interject first a note of caution and then try to redirect the discussion back to a point made by Dr. Stjernswärd. Much of what we have heard, while it is intensely exciting, lacks control groups in certain areas. For example, you mentioned Dr. Sokal's work, which I think is very interesting, but to the best of my knowledge all the patients in that particular work were receiving the manipulation, whatever it happened to be. There was not a matched group not receiving the manipulation.

**Klein:** I understand that there was a matched group not receiving immunotherapy. They were all receiving other therapy, just like Dr. Mathé's group.

**Terry:** I will stand corrected on that one, though I would like to see the published data on that.

Let us go back to what Dr. Stjernswärd tried to pick up, particularly in your own work which is obviously of very great interest. Under what circumstances do you see distant effects? How many of those effects are in excess of an inflammatory reaction at a distant site? And what do you think—or what does anybody think—are the mechanisms involved here?

**Klein:** We cannot fully answer your question, but certainly it is not a coincidence that Dr. Morton, Dr. Thomas, I, and other people do see distant effects. To be sure, all these diseases are variable, and all these diseases have had "spontaneous" regression of tumors in distant, totally untreated, regions.

Now the question is: To what extent are we helping to induce these disappearances? If they occur within 24 or 48 hours after you produce the reaction, I think it is worth following through to find out what the mechanism is, which to be sure, we don't know. Any comment?

**Morton:** The incidence of spontaneous regression in malignant melanoma is <1%, so any time that you can show a regression rate of 20% or 25%, that is much above any reported figures of spontaneous regression in malignant melanoma.

Secondly, when we initially reported our findings we believed that there were 2 mechanisms, nonspecific and specific. The nonspecific effect of the induction of the delayed-hypersensitivity reaction within the melanoma nodule is that the melanoma cells were believed to be killed as "innocent-bystander" cells because no reactions were seen except in patients who became tuberculin positive.

Now in addition to that, to explain the 20% regression of uninjected nodules, we assumed there was a specific effect because we had evidence of a rise in antimelanoma antibodies. That is, what we were doing was raising the patient's systemic immunity and therefore the patients were having regression of uninjected lesions.

After seeing Dr. Zbar's data on how BCG goes all over the body, it may very well be that, when we put the BCG into the melanoma nodule, it also goes to melanoma nodules some place else in the skin. Therefore, it may be that the whole effect is one of a nonspecifically induced, "innocent-bystander" regression.

**Klein:** I would like to add that the same relates to PPD. Dr. S. Landi of the Connaught Laboratories has data on

<sup>14</sup>C-labeled PPD. After PPD is administered—Dr. Landi, if I am correct—within 15 minutes 95% of it is in the bloodstream. So clearly the PPD goes all over the body. Now whether that is the reason for it, we don't know; but the hypothesis is reasonably good.

Furthermore, I think the Rapp-Zbar system clearly indicates that, when you do something at the tumor site, the metastases in the regional lymph nodes disappear. Clearly, there is a distant effect which can be demonstrated by well-controlled animal studies and by less well-controlled human studies.

**Wepsic:** Dr. Zbar, do you have evidence of metastatic cells in locations other than the lymph node when you do your immunotherapy?

Dr. Klein, to what extent can you use steroids to quiet the inflammation, and do steroids affect the lesions?

**Zbar:** When the animals are treated, metastases are not limited to the lymph node but are also present in more distant lymph nodes, such as the axillary lymph nodes which are also eradicated.

**Klein:** Dr. Wepsic, we can turn the reaction off with systemic or topical steroids. Nevertheless, the tumors continue to disappear, clearly indicating that once the trigger is pulled, the process continues.

**M. M. Sigel:** I will just finish up the second part. In the figure you showed on a blastogenic transformation of the lymphocyte, was this done with hapten or conjugated protein?

**Klein:** It was done with hapten.

**Mavligit:** Dr. Humphrey, there is mounting evidence that soluble antigen may escape the processing by macrophages and go directly to expose itself to B-cells and then turn out enhancing antibodies. How closely did you look for enhancement? What tests did you do to ensure that you did not enhance any of those patients?

**Humphrey:** It is a clinical judgment, obviously. The whole problem is that we don't have methods of specifically putting our finger on what goes on serologically. There has been no evidence of sudden increase of tumor growth in any patient.

**Klein:** I would like to ask one more question of Dr. Baldwin because the data on systemic regressions of multiple malignant lesions in the lung were of enormous significance.

**R. W. Baldwin:** These are preliminary experimental data at the present. A chemically induced sarcoma was injected intravenously and became implanted in the lungs. One could treat these tumors and get regression of pulmonary lesions in two ways. One could implant a mixture of viable sarcoma cells with BCG subcutaneously, or one could give a single intravenous injection of BCG and get regressions of lung metastases.

There is just one second point. In these model systems, if you use immunogenic tumors, this system will work. If you use nonimmunogenic tumors, it won't work.

**Klein:** This obviously will require more elaboration.

**D. M. Mumford:** What adverse reactions have occurred from the immunotherapeutic trials?

**Morton:** We have had ulcerations at the injection site, fever, nausea, and vomiting. We don't consider those reactions very adverse. However, there are 2 types of very dangerous reactions. One is an anaphylactoid reaction that

we have seen which has been very severe in a patient receiving multiple injections of BCG. The second type is systemic BCG infection. We have had at least 3 patients with this reaction. One patient required prolonged drug therapy and was very ill.

**Klein:** Would you comment? Did you use isoniazid?

**Morton:** Yes, we used everything.

**Klein:** Did you use other antitubercular therapy?

**Morton:** Yes.

**Klein:** We have heard enough to know that there is something going on that helps the patient. We don't know how much. We have got a long way to go.





# Alpha-1-Fetoprotein: Serologic Marker of Human Hepatoma and Embryonal Carcinoma<sup>1, 2</sup>

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**SUMMARY**—Alpha-1-fetoprotein (AFP), an embryo-specific  $\alpha$ -1-globulin, is the first specific serologic marker of a human malignancy to be in routine clinical use. It is detectable in the serum of 50–100% of hepatoma patients, depending on the ethnic origin of the patient and the sensitivity of the assay used. Sensitivity has been further augmented by <sup>125</sup>I-labeled crossed antibody electrophoresis, which increased the frequency of AFP-positive hepatoma sera. AFP was also detectable in most patients with hepatoblastoma, in one-third of patients with embryonal teratoblastomas, and in 7% of 28 patients with metastatic liver carcinoma. The quantitative levels of AFP were useful in detecting residual hepatoma after total or partial hepatectomy and in following the course of patients with teratoblastoma. In addition, AFP was detected transiently in the sera of 3 patients with viral hepatitis. Both AFP and hepatitis-associated antigen were detected transiently in only 1 of the 50 patients with nonviral hepatic necrosis. AFP is not a product of liver regeneration, since it was not detectable in 7 patients after hepatic lobectomy with active liver regeneration.—*Natl Cancer Inst Monogr* 35: 415–420, 1972.

THE SEROLOGIC detection of human tumor-specific antigens is now a practical reality for some tumors. Alpha-1-fetoprotein (AFP) is the first such serologic marker to be used clinically. Although AFP was known to be a fetal-specific alpha-1 globulin since 1944 (7), it was not until 1963 that Abelev made the significant discovery that AFP was a carcinoembryonic antigen (2). His observation, that

a tumor-specific hepatoma antigen extracted from mouse hepatoma cells was immunologically identical to a normal mouse fetal-specific serum protein, was rapidly confirmed in a variety of species, including man (3). This was the first convincing demonstration of the embryonal “retroversion” or “derepression” of malignant cells and has led to the discovery of other carcinoembryonic antigens that have great potential clinical application. This paper briefly reviews the normal biology of human AFP and describes some of its current clinical applications.

## NORMAL BIOLOGY OF AFP

AFP is not strictly a tumor-specific antigen in man for the following 3 reasons. 1) No autologous human antibody is demonstrable. 2) There is no apparent host immune response in hepatoma to AFP. 3) Despite the recent demonstration of AFP in some maternal sera near term (4), no maternal antibody to AFP has ever been found.

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AFP is synthesized by normal embryonal hepatocytes (5), is secreted into serum, and reaches a peak concentration during the first trimester (6). In early fetal life it accounts for 90% of the total serum globulin. Although the physical and chemical characteristics of AFP are similar to those of albumin (7), the function of AFP in this early embryonic period is still unknown. Serum concentrations fall rapidly during gestation to low levels of 1–5 mg/100 ml in the newborn period and tend to disappear with a half-life of approximately 3 days (6).

We studied 119 consecutive infants admitted to the Pediatric Service of the Massachusetts General Hospital (*see* table 1). All patients under 4 weeks of age had detectable AFP, falling rapidly in concentration after birth. Most of the infants 4–8 weeks old had low levels (<0.1 mg/100 ml). The one patient in this group with active liver disease had the highest AFP level of the group, 2.2 mg/100 ml. After 8 weeks of age, only 3 patients had detectable AFP. All 3 had active liver necrosis—2 from acute hepatic congestion due to congenital heart defects and the third from biliary atresia. Two older infants, with active liver necrosis from biliary atresia and chronic active hepatitis, did not have detectable AFP. Thus AFP may be an acute phase-reactant protein in infants up to approximately 11 or 12 weeks of age, or preformed AFP may have been released from liver cells undergoing necrosis in the early postpartum period.

DIAGNOSTIC VALUE OF AFP

Demonstrable AFP in children after approximately 3 months of age is highly suggestive of 1 of

3 specific childhood malignant tumors. We have detected AFP in all 6 infants tested with hepatoblastoma, as well as in most children with hepatic carcinoma. AFP was reported in 37% of children with embryonal carcinoma of the gonads (8).

The reappearance of AFP in the sera of adult patients with hepatoma has been well documented since 1964 (3). Shown experimentally to be synthesized by malignant hepatocytes (2), as well as by embryonal liver cells (5), AFP was found in 50–95% of several large series of hepatoma patients and proved to be useful clinically in the diagnosis of this frequently occult tumor.

The frequency of AFP-positive hepatoma patients appears to vary with the geographic origin of the patients (9, 10). This geographic variation was consistent, despite the increase in sensitivity by over 50-fold, with the detection of additional hepatoma patients (11). Although the biology of liver cancer in the high-incidence areas of Africa and Asia may be somewhat different (11) than that in the United States, the reason for this geographic variation of AFP is still unknown.

The serum concentration of AFP varies widely (11, 12). Therefore, the frequency of AFP in hepatoma patients will also depend on the sensitivity of the assay system (table 2). Double-gel diffusion, with a sensitivity of 1–10 µg/ml (11, 12), detected approximately 30% of Caucasian and 50–90% of non-Caucasian hepatoma patients (8–16). Modified versions of counter-immunoelectrophoresis, with a sensitivity of 200–250 ng/ml, significantly increased the frequency of AFP-positive hepatoma patients (11). We have achieved even greater sensitivity by electroimmunodiffusion in dilute rabbit antibody and subsequent incubation with high-

TABLE 1.—AFP in infants

Age	Number of infants	Positive		Infants with active liver disease	AFP
		Number	Percent		
0–4 wk	18	18	100		
4–8 wk	7	5	71.4	8 wk, biliary atresia	2.2 mg/100 ml
8–12 wk	7	3	33.3	10 wk, acute hepatic congestion	Trace
				10 wk, acute hepatic congestion	Trace
12–24 wk	10	0	0	11 wk, biliary atresia	Trace
24–52 wk	16	0	0	13 wk, biliary atresia	ND*
1–2 yr	61	0	0	9 months, chronic active hepatitis	ND*
	119				

\* Not detectable.

TABLE 2.—Sensitivity of AFP assays

	Lower limit of sensitivity ( $\mu$ g/ml)	AFP + hepatomas
1) Double-gel diffusion	1.0-10	30 to 50-90%
2) Counter-immuno-electrophoresis	0.20-0.25	50 to 75-95%
3) Radio-labeled, crossed antibody electrophoresis	0.05-0.10	?
4) Competitive-inhibition radio-immunoassay	?0.001-0.010	? 100% ? normals

specific activity,  $^{125}$ I-labeled, goat anti-rabbit IgG. Subsequent radioautography on X-ray film (fig. 1) could detect as little as 50-100 ng/ml. Using this method, we detected AFP in only 1 of 15 hepatoma patients who were AFP negative by counter-immuno-electrophoresis. We have still been unable to detect AFP in normal adults or in patients with regenerating liver after hepatic lobectomy.

Recently, AFP was reported in trace amounts in normal human serum and in 100% of sera from hepatoma patients by means of a competitive-inhibition type of radioimmunoassay (17, 18). However, the specificity of such a system depends on the degree of purity of the radio-labeled, purified AFP. In addition, nonspecific competition with antigen binding would simulate trace levels of AFP. Therefore, whether the repression of synthesis of this normal fetal protein is almost or totally complete with maturation in adult life is still unresolved. Nevertheless, the direct demonstration of AFP by various forms of immunoprecipitation in clinical use has proved a reliable and useful serologic test for primary liver cancer.

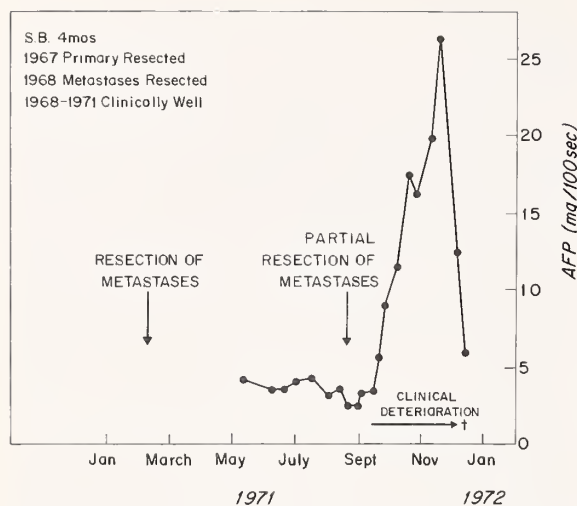
Several recent case reports have documented the presence of AFP in the serum of a few patients with carcinoma of the stomach and pancreas metastatic to the liver (15, 19-22). Consistent with this observation was the demonstration by Gitlin of fetal gut synthesizing trace amounts of AFP (23). We studied a series of patients with gastrointestinal tumors metastatic to the liver to determine the frequency of AFP in metastatic carcinoma. To date, we have found 2 of 28 (7%) metastatic carcinomas to have trace amounts of detectable AFP by modified electroimmunodiffusion: one a gastric carcinoma and the other a pancreatic carcinoma, both with large metastases.

## PROGNOSTIC VALUE OF QUANTITATIVE AFP LEVELS

The quantitative levels of serum AFP vary widely from several hundred ng/ml to several hundred mg/ml (11, 12). There is no significant correlation between the absolute serum concentration and any clinical or pathologic criteria that have been examined. The levels tend to be relatively stable, with some general tendency to increase with variable slopes in individual patients. Occasionally in a patient near death, serum concentration falls precipitously (11, 12). Nevertheless, a change in serum concentration has been shown to be extremely useful prognostically after lobectomy (24) or orthostatic transplantation (25). In addition, rising levels of AFP have preceded clinical recognition of recurrence of hepatoma or growth of metastasis (25). We studied 2 children with localized hepatoblastoma, thought to be cured by hepatic lobectomy; persistence of low levels of AFP in these children suggested residual tumor, which eventually became clinically obvious. When better therapy becomes available for hepatic carcinoma, AFP can be used prognostically as a serologic marker of treatment.

Another tumor, in which the diagnostic usefulness of AFP has been documented, is embryonal carcinoma of the gonads, a particular variety of childhood tumor. AFP was found in 37% of children with this tumor (8). We recently found low levels of AFP in 2 adults, a 21-year-old female with ovarian embryonal carcinoma and a 28-year-old male with primary retroperitoneal embryonal carcinoma and no gonadal involvement. In this tumor, the change in serum concentration can be also used prognostically (text-fig. 1). In collaboration with the Oncology Division of the Denver Children's Hospital, we followed a 4-month-old child with embryonal carcinoma of the testis, which was removed surgically in 1967. A retroperitoneal metastasis was partially removed in 1968, and he received postoperative radiation and chemotherapy for 2 years with a complete clinical remission. In January 1971, a recurrent retroperitoneal metastasis was removed, and his preoperative positive AFP test became negative. However, several months later, he developed some nonspecific gastrointestinal symptoms, and his AFP was again detectable. Due to the persistent nonspecific gastrointestinal symptoms and the persistent AFP levels,





TEXT-FIGURE 1.—Serial AFP concentrations in a boy with recurrent metastatic embryonal carcinoma of the testis (in collaboration with the Oncology Service of the Denver Children's Hospital).

he was given chemotherapy and finally was explored surgically. A retroperitoneal recurrence of embryonal carcinoma, suspected largely due to the persistent AFP level, was found and was partially removed. He was placed on chemotherapy, but several months later, as his AFP level began to climb, he began to deteriorate clinically. The AFP level peaked and then precipitously fell just before his death from recurrent embryonal carcinoma.

A second patient, studied in collaboration with the Oncology Division of the Lemuel Shattuck Hospital, was an 18-year-old female with embryonal carcinoma; she was also AFP positive. After the primary ovarian tumor was removed, the AFP concentration diminished considerably but still remained positive. The patient had residual tumor, did not respond to chemotherapy, and died. Hence the serum concentration of AFP in embryonal carcinoma, as in hepatic carcinoma, also appeared to reflect therapy and can be used as a serologic marker for the surgical treatment or chemotherapy of this tumor.

## AFP IN VIRAL HEPATITIS

AFP also is found rarely and transiently in acute viral hepatitis. In 1968, we reported one patient with biopsy-proved viral hepatitis who was AFP positive. But at that time, we were unsure of the

significance (14). However, recently Geffroy *et al.* (26) and Smith (27) reported trace amounts of AFP in the sera of a few patients with acute viral hepatitis. Hersch and Bajaj (28) showed the immunologic identity of the transiently appearing AFP in a patient with viral hepatitis to AFP found in fetal and hepatoma sera (28). Figure 2 shows a similar line of complete immunologic identity, after counter-immunoelectrophoresis, between AFP in viral hepatitis and hepatoma sera. We re-examined the frequency of AFP in a large group of patients with active liver disease by the more sensitive technique of modified electroimmunodiffusion and radioautography (table 3). We found 4 patients positive in 187 samples from 102 patients with active liver disease. Three were positive for hepatitis-associated antigen (HAA). AFP was present transiently in 2 of the 46 patients with acute viral hepatitis and 1 patient with HAA-positive chronic active hepatitis. Both AFP and HAA were found transiently in an 18-year-old patient with nonviral massive hepatic necrosis due to carbon tetrachloride intoxication. All other patients with toxic hepatitis or alcoholic hepatic necrosis were negative for both AFP and HAA.

The transient reappearance of AFP in viral hepatitis does not appear to be related to rapid liver regeneration. We studied 38 early postoperative serum specimens from 7 patients with active liver regeneration following lobectomy for benign diseases; we were unable to detect AFP. Therefore, the reappearance of AFP in non-neoplastic disease appears to be virtually limited to viral hepatitis and perhaps to HAA. In view of the association of HAA- and AFP-positive liver cancer in some parts of the world (29), it is intriguing to speculate that

TABLE 3.—AFP in active liver disease

Liver disease	Number of samples	Number of patients	AFP + patients
Acute viral hepatitis			
HAA positive	68	32	1
HAA negative	41	14	1
Chronic active hepatitis	8	6	1*
Massive hepatic necrosis	15	10	1*
Toxic hepatitis (drugs)	21	10	0
Active cirrhosis with jaundice	34	30	0
	<hr/> 187	<hr/> 102	

\* HAA positive.

the hepatitis virus turns on the embryonal genome for AFP synthesis as part of its oncogenic potential.

In conclusion, AFP is a clinically useful serologic marker of 2 malignancies. First, it is helpful diagnostically by being detectable in 50–95% of hepatomas and in 37% of embryonal carcinomas. Second, it is helpful prognostically, in that the change in serum concentration appears to reflect therapy. Last, AFP is detectable in trace amounts, in perhaps as much as 5–10%, of other entodermally derived gastrointestinal tumors metastatic to the liver. The significance of the transient reappearance of AFP in some patients with viral hepatitis remains to be determined.

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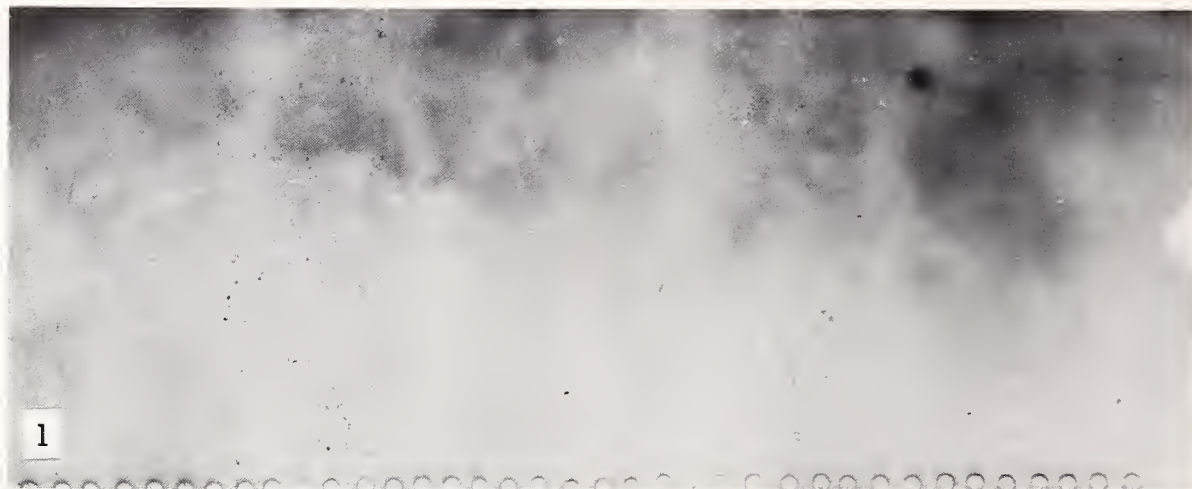


FIGURE 1.—Electroimmunodiffusion in dilute rabbit anti-AFP, with subsequent incubation with  $^{125}\text{I}$ -labeled goat anti-rabbit IgG and development by radioautography on X-ray film. Serial dilutions of AFP-containing serum are seen as labeled rocket precipitates.

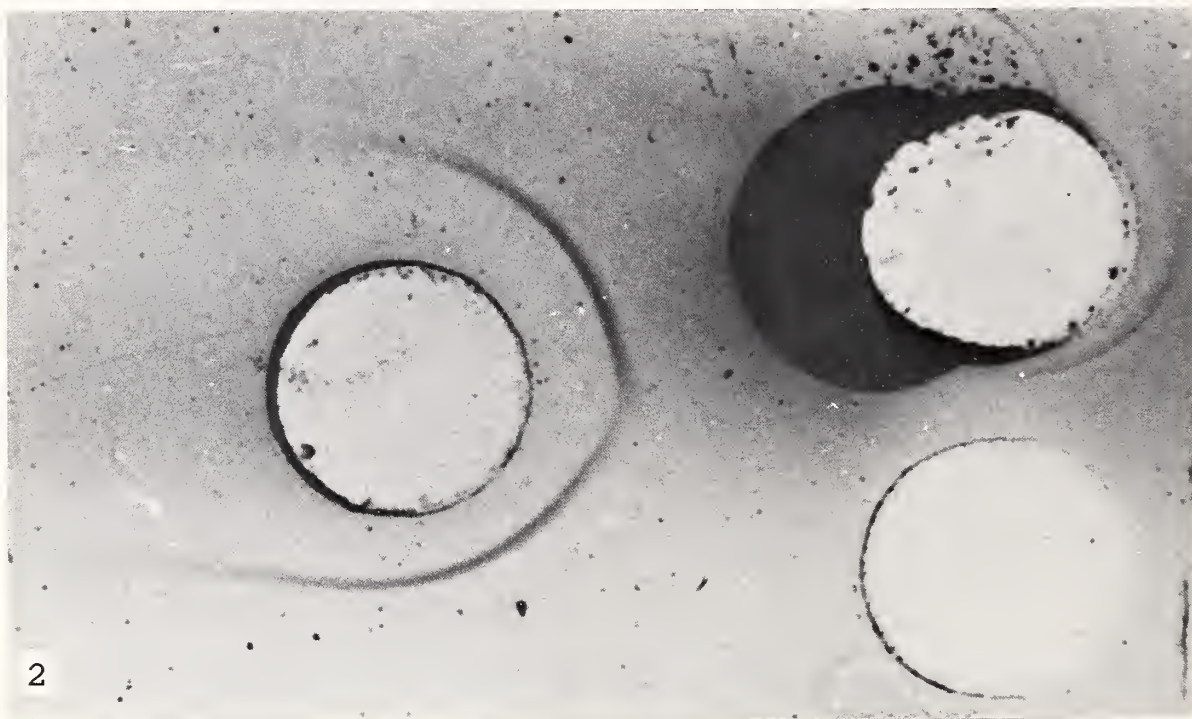


FIGURE 2.—Immunologic identity of AFP in serum of patient with viral hepatitis (*upper well on right*) and hepatoma (*lower well on right*). Counter-immunoelectrophoresis was performed with monospecific rabbit anti-AFP (*well on left*).



# Further Studies on Carcinoembryonic Antigens<sup>1</sup>

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**SUMMARY**—Our studies on carcinoembryonic antigens (CEA) deal with several points still obscure or unknown. The cancer specificity of CEA is discussed. CEA was found in many noncancerous colonic mucosae. A possible correlation between the CEA synthesis and the rate of proliferation is under investigation. The membrane localization of the CEA is a rule that has some exceptions: Cytoplasmic localization was found in some gastric cancers. A protein able to cross-react with the CEA was characterized and named “nonspecific cross-reacting antigen,” or NCA. It is also a perchlorosoluble  $\beta$ -globulin, found in colonic mucosa, lung, spleen, and plasma. The possible consequences of this cross-reacting protein for the CEA radioimmunoassay are discussed.—*Natl Cancer Inst Monogr* 35: 421–425, 1972.

SINCE THE discovery of carcinoembryonic antigens (CEA) (1) much work has been devoted to this antigen to answer several important questions: Is the CEA really cancer specific? If not, what is its significance? Are there CEA-like substances in cancerous and normal tissues?

The cancer specificity of CEA was claimed by Gold and Freedman (1) when they discovered this antigen. von Kleist and Burtin (2) at first reported similar findings concerning the absence of CEA from normal colonic mucosa. More recently Coligan *et al.* (3), using a sensitive radioimmunologic method, were unable to find CEA in normal colonic mucosa.

However, some results cast doubt on these findings. First Martin and Martin (4) absorbed an anti-CEA antiserum with very high amounts of perchloric extracts of some noncancerous colonic mucosae.

Furthermore, immunofluorescence studies in our

laboratory (5–7) showed CEA was in many noncancerous colonic mucosae, with the same centroglandular localization as in colonic tumors.

In our first experiments, CEA was seen in histologically normal pericancerous mucosae (5). This result did not surprise us, since other biochemical abnormalities already had been described in pericancerous colonic mucosa, *i.e.*, changes in the rate of synthesis of nucleic acids and in the pattern of lactic dehydrogenase isoenzymes—alterations less pronounced, but similar to those obtained in cancerous glands. Later we examined polyps of the colon and the rectum by immunofluorescence (6). The 25 polyps studied were of different histologic types, *i.e.*, either benign, premalignant, or containing a carcinoma *in situ*. All reacted with anti-CEA antiserum. The intensity of the fluorescence varied in relation to the level of dedifferentiation, but not in relation to the histologic type of the polyps. In a given polyp, the relatively differentiated glands reacted more strongly than the undifferentiated ones, and benign polyps were as positive as premalignant ones.

The presence of CEA in polyps was evident in a study of a perchloric extract prepared from a pool of 9 benign polyps. In these extracts we character-

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ized the antigen, which, in Ouchterlony plates, gave an identity reaction with CEA extracted from colonic carcinomas.

CEA was also found by immunofluorescence in the colonic mucosa of patients afflicted with ulcerous colitis and in hemorrhoidal mucosae (6). Many samples of the latter were examined, and results varied. When stained by anti-CEA antiserum, some hemorrhoidal mucosae gave a bright fluorescence. They contained slightly dedifferentiated glands surrounded by inflammatory infiltrates. Others of normal histology were weakly positive or even negative. In these cases, the fluorescence was sometimes more intense in the proximal than in the distal part of the glands. It could sometimes be located only on the surface epithelium and the isthmus of the glands. On the contrary, strong fluorescence was equally distributed over the whole length of the glands, as in tumors.

More recently we studied the colonic mucosae of infants and young children up to the age of 7 years (7). The 48 samples we examined by immunofluorescence were all positive. The intensity and distribution of fluorescence varied, which was unrelated to the age of the children. Moreover, extracts made from these mucosae with phytic acid (used instead of perchloric acid because it is less denaturing) contained CEA identical in Ouchterlony plates to the reference CEA prepared from colonic tumors. Thus in the young, CEA is a normal component of the colonic mucosa.

According to these findings, it does not seem possible to consider CEA as cancer specific, as far as the colonic mucosa is concerned. Here the real significance of CEA has still to be discussed. Can it be found in really normal colonic mucosa, even in the adult? Or is its presence related to an inflammatory process, eventually responsible for a higher proliferation rate of the epithelial cells? To answer the first question, we recall our findings and those of others who were unable to characterize CEA in the adult normal colonic mucosa examined. We are actually studying the second point. We try to correlate the amount of CEA on the membrane of the epithelial crypts of hemorrhoidal mucosae with the rate of cell proliferation in the same glands. For this purpose, we use a combination of two methods:

1) Incorporation of tritiated thymidine *in vitro* into fragments of hemorrhoidal mucosae, followed by autoradiography. 2) Detection of CEA by

immunoperoxidase staining. This technique was used instead of immunofluorescence because results given by immunohistology and autoradiography could be compared on the same slide; immunofluorescence patterns must be read before autoradiography because the development of the photographic emulsion inhibits the emission of the fluorescence. The results obtained are too preliminary to be discussed.

The study of CEA in gastric mucosa gave results different from those obtained with colonic mucosa (8). We rarely found this antigen, by immunofluorescence, in the noncancerous mucosa, *i.e.*, of stomachs excised for ulcers, both gastric and duodenal. In the histologically normal area surrounding gastric tumors, CEA was seldom observed. In gastric carcinomas, CEA was almost always present. Most often it was at the apical pole of epithelial cells, thus bordering the lumen of the cancerous glands as in colonic tumors. But, there were exceptions to this pattern. Sometimes only part of the cancerous gland was stained by CEA antiserum, most of the glands being negative. This last result was obviously different from those results obtained in colonic cancer, where all the tumorous glands contained CEA. In some undifferentiated gastric tumors, having lost their glandular organization, CEA was found at the periphery of the invasive cancerous cells: It was either associated with the cell membrane, formed a pericellular deposit, or was located, at least partially, in the cytoplasm of the cells. In cases of linitis plastica, cytoplasmic structures were seen which were stained brightly by anti-CEA antiserum. These last findings showed that CEA is not always associated with the cell membrane in gastric cancer.

Another problem is the possible existence of CEA-like substance(s) in tissues other than digestive cancers and eventually in digestive cancers themselves. Preliminary reports (9-11) indicated such a substance related to CEA in normal lung or spleen extracts.

A few months ago, we successfully isolated and characterized in various tissues an antigen cross-reacting with CEA.

Several years ago, we described in perchloric extracts of colonic tumors, besides CEA, a  $\beta$ -globulin, the precipitin line of which was always nearer the antibody trough than that of CEA (2). The component giving this precipitin line was separated



from CEA by filtration on Sephadex G-200. When studied by immunodiffusion in gel with an antiserum against purified CEA, it gave a typical cross-reaction with CEA (fig. 1). The same antigen was found in perchloric extracts of normal colonic mucosa, lung, spleen, and in very low amounts in liver (fig. 2). It was neither organ nor cancer specific. For these reasons (lack of cancer and organ specificity and cross-reaction with CEA), we named this component "nonspecific cross-reacting antigen," or "NCA" (12).

NCA is a glycoprotein because its precipitin line is stained by the periodic acid-Schiff reagent. It supposedly has a molecular weight lower than that of CEA: According to a preliminary result, it has a sedimentation constant about 3.5S. It is more sensitive to acidic pH than CEA: Thus it is less abundant in perchloric extracts prepared by Gold's method, which are not neutralized before dialysis, than in our perchloric extracts, which are neutralized right after centrifugation.

NCA has a complex antigenic structure. As mentioned, it gives a cross-reaction with CEA when reacted with an anti-CEA antiserum. Conversely, when studied with an antiserum against normal colonic mucosa, it spurs over CEA. Thus both antigens have a common antigenic structure (or determinant), and each of them has a specific one. The chemical nature of these structures is yet to be determined.

According to these findings, NCA clearly is not a fragment of CEA, since it bears an antigenic determinant not found in CEA.

We also found NCA in normal plasma and serum (13). We prepared perchloric extracts of 3 pools and 5 individual samples of plasmas and of a pool of sera obtained from healthy individuals of various blood groups. All these extracts were processed according to our method, which includes the neutralization step just after the centrifugation. Another pool of plasma was extracted by perchloric acid and not neutralized before dialysis, as described by Gold. All these extracts, after a strong concentration (from 300 ml to 1–2 ml) in Ouchterlony plates gave a precipitin line joining that of NCA and cross-reacting with CEA (fig. 3). We do not know yet if these results may be generalized, but it seems likely that NCA is a normal component of human plasma.

The fact should be stressed that antisera, even obtained in animals immunized with the most

purified CEA, may contain antibodies able to cross-react with NCA. Our antisera contained them, in proportion to their titer in anti-CEA antibodies. Before an antiserum is used against CEA, it should be studied for the presence of antibodies able to cross-react with NCA. One can do this by reacting it not only with purified CEA, but also with perchloric extracts of colonic tumors, normal colonic mucosa, and lung (or spleen) (fig. 4). If such antibodies are found, they should be absorbed out, either with purified NCA or with perchloric extracts of lung (or spleen).

Consequently, studies showing the presence of CEA in noncancerous tissues can be considered valid on two criteria only:

- 1) Isolation of the antigen from tissues, and immunochemical demonstration for its identity with CEA isolated from colonic tumors. We obtained this evidence in our studies on the polyps of the colon and on colonic mucosae of infants and young children. In both cases, the presence of CEA was firmly established. In other works this has not been so.

- 2) Use in immunofluorescence studies of an antiserum carefully absorbed out until all the cross-reacting antibodies disappear. We used such a reagent in our work on ulcerous colitis and hemorrhoidal mucosa. It is true that we know about the existence of only one NCA, and we can absorb out the antibodies able to react with it. But existence of other components able to cross-react with CEA cannot be ruled out.

In the same way, the use of an antiserum deprived of its cross-reacting antibodies seems indispensable for the radioimmunoassay of CEA, since NCA is present in many normal plasmas and, logically, also in pathologic ones.

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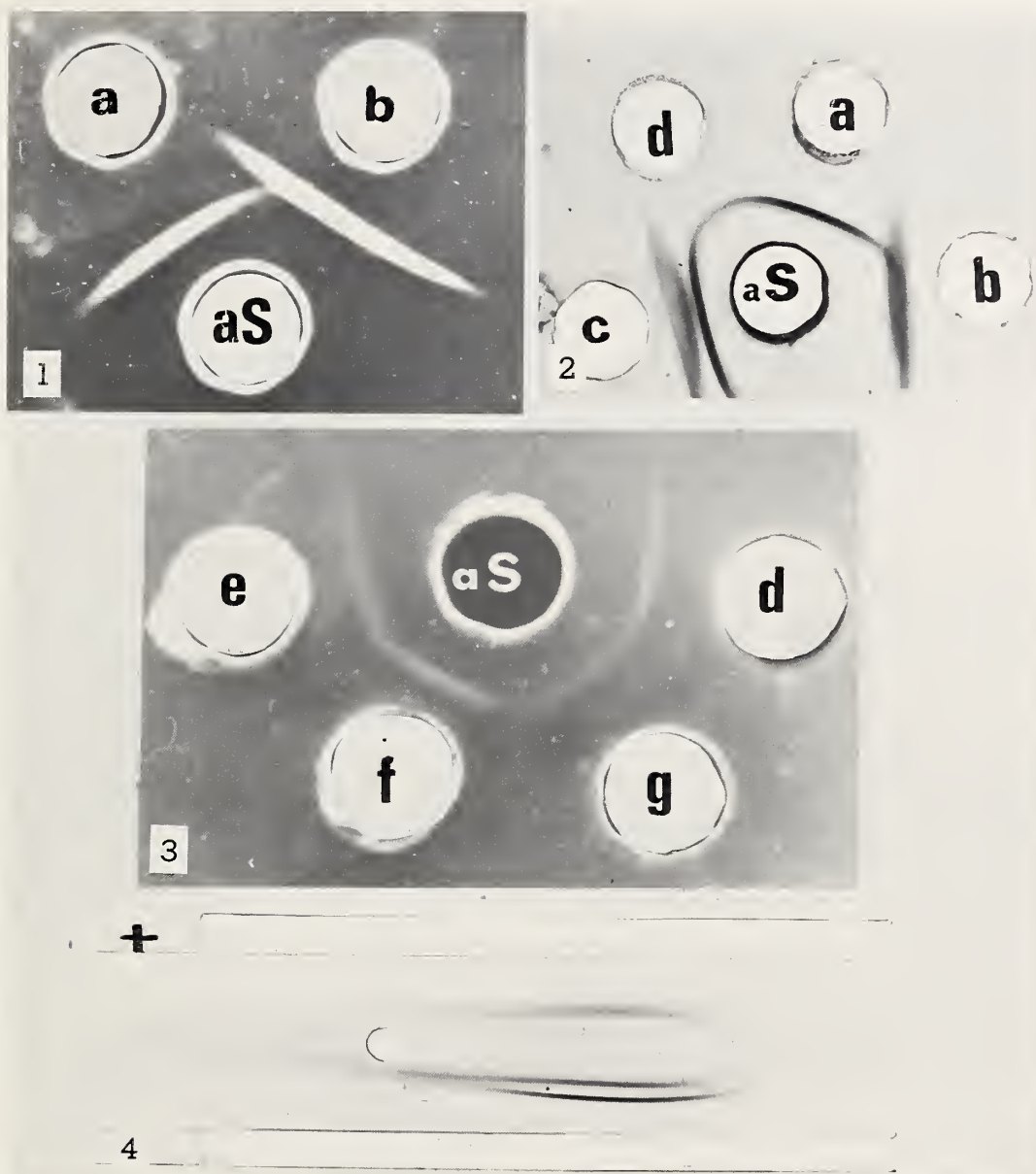


FIGURE 1.—Ouchterlony plate showing a partial identity reaction between the normal cross-reacting antigen [(NCA) = a], the carcinoembryonic antigen [(CEA) = b], and an anti-CEA antiserum (aS).

FIGURE 2.—Ouchterlony plate showing NCA in semipurified colonic tumor extracts (c); lung semipurified extract (d); NCA (a); and CEA (b); For aS and all other abbreviations *see* figure 1.

FIGURE 3.—Ouchterlony plate showing NCA in spleen (e) and normal human plasma neutralized extracts (f) in unneutralized human plasma extract (g) and lung semipurified extract (d). aS as in figure 1.

FIGURE 4.—Immunoelectrophoresis of a colonic-tumor semipurified extract. At *left*, an anti-CEA antiserum, which contains cross-reacting antibodies; at *right*, same antiserum absorbed by a semipurified lung extract. Only the CEA precipitin line is left. The negative pole is on the *bottom*.





# Detection of Carcinoembryonic Antigen by Radioimmune Assay<sup>1, 2</sup>

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**SUMMARY**—Radioimmune assays were developed for the detection of carcinoembryonic antigen (CEA) and were used in examination of a variety of biologic materials. They also facilitated the isolation of material for structural studies and enabled a study of the production of CEA in tissue culture. Radioimmune assays are considered in terms of their potentials and limitations for cancer detection.—*Natl Cancer Inst Monogr* 35: 427–432, 1972.

ALTHOUGH the carcinoembryonic antigen (CEA)<sup>3</sup> was first described by Gold and Freedman in 1965 (2), great impetus was given to its study in 1969 by the publication of a method for a sensitive radioimmune assay capable of detecting circulating CEA in human sera (3). A modification of this assay employing zirconyl phosphate instead of ammonium sulfate to precipitate the antibody-CEA complex was subsequently described (4). Studies with these assays indicated that the measurement of CEA might prove useful for the detection and diagnosis of various malignancies and for following therapy.

## DOUBLE ANTIBODY ASSAY FOR CEA

In our laboratory we undertook the development of a radioimmune assay for CEA in the serum. For use in surveys for detection of cancer, such a test should be highly sensitive, reliable, and readily applicable to the examination of large numbers of sera. Previous tests had utilized comparatively large amounts of sera or plasma and involved multiple operations, such as dialysis and lyophilization.

The test we developed is performed directly on 0.2 ml of serum in a single disposable tube without any change of container. Since this test was described previously (5–8), only a brief summary is presented here (text-fig. 1).

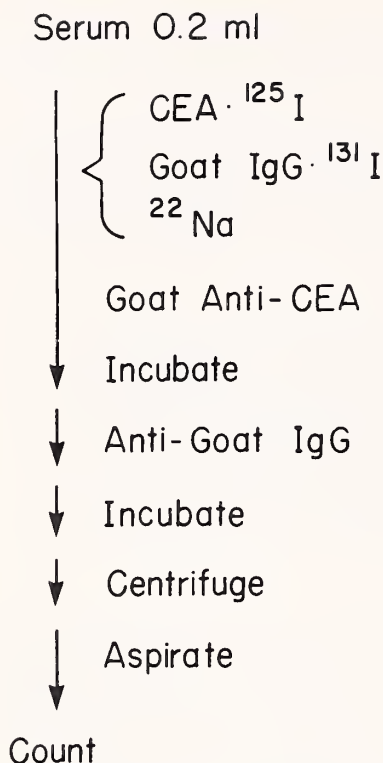
The assay employs the double antibody technique (9, 10) and utilizes 3  $\gamma$ -emitting radioisotopes. The CEA-<sup>125</sup>I indicates the competitive binding of serum CEA with the added indicator CEA-<sup>125</sup>I. The rabbit IgG-<sup>131</sup>I monitors the reproducibility with which the radioactive mixture is added and provides assurance that the precipitation of anti-CEA by the secondary antibody is complete and that none of the precipitated material was inadvertently removed during the aspiration immediately preceding the final counting. <sup>22</sup>Na indicates the amount of supernatant left after its nearly complete aspiration (11). From this information it is possible to apply a correction for residual supernatant. Thus, washing of the precipitate is unnecessary, and a portion of the supernatant is intentionally left on the precipitate to minimize the risk of removing precipitate in the aspiration step. The radioactivity is determined with a Nuclear Chicago crystal scintillation counter equipped with a 512 channel analyzer and a multiple region of interest accessory. The data are recorded on punched tape for analysis by a Wang calculator.<sup>4</sup>

<sup>1</sup> Presented at Conference on Immunology of Carcinogenesis, held by the Oak Ridge National Laboratory at Gatlinburg, Tenn., May 8–11, 1972.

<sup>2</sup> Supported in part by Public Health Service grant CA12631 from the National Cancer Institute and by the Stuart L. Bernath Cancer Research Fund.

<sup>3</sup> The literature on CEA has recently been reviewed by Gold (7).

<sup>4</sup> A program has been written to perform these calculations on a Wang 700 calculator equipped with a 703 paper tape editor and 702 output writer. This program is available on request to the authors.



TEXT-FIGURE 1.—The triple isotope, double antibody assay for CEA.

The double antibody technique allows us to detect 0.5 ng in 0.2 ml of serum or 2.5 ng/ml.

### IMMUNE ADSORBENT ASSAY FOR CEA

The elapsed time for the double antibody assay from start to final counting is 5 hours. There are 2 incubation periods separated by the operation of pipetting the secondary antiserum. This elapsed time can be further shortened by the use of a solid immune adsorbent, which obviates this pipetting step and eliminates the second incubation (text-fig. 2).

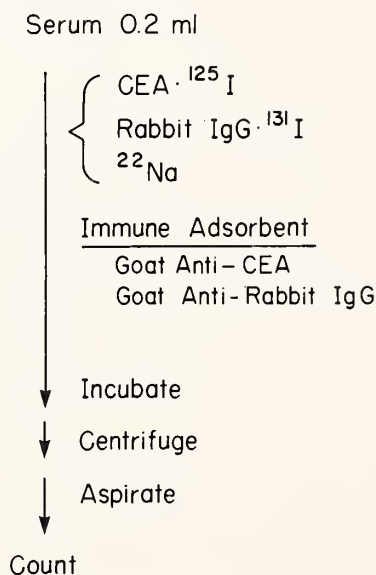
In its present form, we are using for the immune adsorbent assay goat anti-CEA serum and goat anti-rabbit IgG serum individually insolubilized by treatment with ethyl chloroformate (12). Subsequent to the insolubilization, the 2 immune adsorbents are mixed in an appropriate ratio. In the assay, rabbit IgG·<sup>131</sup>I is added with the CEA·<sup>125</sup>I and <sup>22</sup>Na to correct for residual supernatant. The concentration of the goat anti-rabbit IgG immune adsorbent is adjusted to bind 50 % of the rabbit

IgG·<sup>131</sup>I (approximately 1 μg/tube). An increase in the amount of rabbit IgG·<sup>131</sup>I bound indicates that the particular serum being tested is causing increased nonspecific binding to the insolubilized immune adsorbent, while a decrease signals nonspecific interference of binding by the serum in question. Performance of the assay is thus monitored by the rabbit IgG·<sup>131</sup>I adsorbed, similar to the use of the goat IgG·<sup>131</sup>I in the previous method.

With a solid immune adsorbent, the assay time is reduced to 3 hours, and handling of the assay tubes is reduced, thus lessening technician time needed for each assay. However, more anti-CEA serum is required, since the antibody-binding sites are apparently used less efficiently in the solid immune adsorbent. The assay in its present form is also less sensitive than double antibody assay, and we are investigating modifications of the insolubilization technique in the hope of improving the sensitivity.

### SENSITIVITY OF RADIOIMMUNE ASSAYS

The sensitivity of a radioimmune assay depends in part on the binding affinity of the anti-CEA serum. Presumably the appropriate protocol for the preparation of the antiserum (13), or good luck, could lead to serum of greater affinity and thus increased sensitivity. At the present state of



TEXT-FIGURE 2.—Assay of CEA by immune adsorbent technique.

our knowledge, it is difficult to know what our goal with respect to sensitivity should be. As a first approximation, one might assume that the more sensitive the test, the earlier it would detect incipient cancer. This assumes that the CEA concentration in the serum is related to tumor size. We do not know if this is true during the development of an individual tumor, but we do know that tumors of the same tissue origin vary considerably in their CEA content (14).

The assay values for CEA in the serum of normal individuals by various techniques seems to be  $\leq 2.5$  ng/ml. Experience with the double antibody assay in England indicates that this level may go as high as 7 ng/ml. Our experience has been that normal serum shows an apparent CEA content of about 2.5 ng/ml, when the assay is referred to a standard curve obtained by use of CEA in buffered saline. When the assay is referred to a standard curve obtained with CEA in normal serum, the value fluctuates around zero. We are undecided at the moment whether this difference reflects a nonspecific change in the milieu of the assay, or alternatively, results from specific material participating directly in antibody binding. The latter may be a material cross-reactive with CEA, or CEA itself. Should the material be indeed CEA, it would appear that increased sensitivity of the assay would let us study variations in the normal low level of CEA in human serum, but would probably not augment our ability to detect cancer.

### SPECIFICITY OF RADIOIMMUNE ASSAYS

The high degree of selectivity characteristic of the immune reaction is the basis of radioimmune assays. This high selectivity can lead to a false sense of security in interpreting assay results. Because of the importance assumed by radioimmune assays in studies of antigens generally, and CEA in particular, it may be well to consider here the fundamental nature of a radioimmune assay and examine some of the influences that can lead to false interpretations.

The essence of a radioimmune assay is the combination of antibody with radio-tagged antigen and the interference of this combination by a substance present in the material being tested. Errors in interpretation can derive from at least 3 sources: 1) adverse influences on antigen-antibody reactions generally, 2) reactions between associated impuri-

ties and their homologous antibodies, and 3) cross-reactions between related materials capable of competition with the true antigen for antibody sites.

Under the first category can be mentioned the influences of pH and ionic strength, as exemplified by the ion-sensitive site described by Lo Gerfo *et al.* (4); the influence of complement as noted in insulin assays by Morgan *et al.* (15, 16); and the presence of an enzyme capable of destroying the reference standard as found by Page *et al.* (17). Because pathologic sera may differ from normal sera in many ways, one must always be alert for interferences of these types.

Fortunately there is a simple clue to the possibility of interference from the second category above, *i.e.*, reactions between associated impurities and their homologous antibodies. Ideally, a radioimmune assay would involve the reaction of an antigen of absolute purity with antibodies directed uniquely against this antigen. In practice, sera rather than purified antibodies are used. Even though such sera may have originated in animals immunized with antigen of high purity, the serum, nonetheless, will reflect many aspects of the past immunologic experiences of the donor animal. Usually the provoking antigen contains to a greater or lesser degree contaminants, particularly if it has been necessary to obtain it by isolation from a biologic source. Small amounts of contaminants may provoke the synthesis of disproportionate amounts of antibodies. Thus it is likely that antisera in general tend to be less pure than their corresponding antigens. As a consequence, if the antigen used as the tagged standard contains a significant amount of impurity,<sup>5</sup> in reality 2 independent assay systems have been established. Either or both of these systems can respond, depending on the antigen(s) present in the material being tested. Normally one would not know from the initial test result whether one or the other, or both, were responsible. Further testing at higher concentrations could be revealing. If only one of the antigens is present, complete inhibition could not be achieved. Such a system might lead to the conclusion that in a given situation the content of antigen never exceeded a fixed amount. If both were present in the material tested, but in a ratio that differed significantly from that of the radio-

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<sup>5</sup> More properly stated—if the impurity contains a significant amount of radioactivity.



tagged antigen, it would not be possible to duplicate the standard curve using the test material.<sup>6</sup> Since in many cases the radioimmune assay will be used to examine material of similar origin to that used for the primary standard, this insidious situation could frequently be present.

Finally let us consider the influence of cross-reacting materials. This situation differs from the one just discussed in that both antigens are competing for the same, or some of the same, antibodies present in the antisera. This competition stems from certain structural similarities between the test antigen and its cross-reactive counterpart, here designated the "cross-reactive material." This cross-reactive material may bind to the antibody with an affinity that exceeds that of the test antigen. In this case, this cross-reactive material will displace greater amounts of the antigen than would an equal amount of the test antigen.

More commonly the cross-reactive material will bind with less affinity than the test antigen. If the cross-reactive material differs substantially in structure from the test antigen, the difference in affinity could be great. Yet, if present in sufficiently high concentration, even cross-reactive materials with quite dissimilar structures might simulate the presence of small amounts of the test antigen. With such a material, one can construct a binding curve. Basically, the shape or slope of the binding curve will be determined by the antibody population. In the simplest case, if the antibody population is essentially homogeneous, the binding curve for the cross-reactive material would be indistinguishable from that for the test antigen, except that higher concentrations would be necessary. Neither identical shapes nor slopes of binding curves indicate identical antigens. A pertinent example has been demonstrated with the nonapeptide, bradykinin, and 4 of its synthetic analogues (18). Each binds with a different affinity, but with an identical slope, to an antiserum to bradykinin.

In a more complex situation, if the cross-reactive antigen were capable of reacting with essentially the same relative affinities as the test antigen with each of the components of a heterogeneous antibody population, the binding curves again would have similar shape and slope. In both of these

cases, complete inhibition could be obtained by the cross-reactive material. On the other hand, if the cross-reactive material could react with some, but not all, of the antibody population, the slope of the binding curve would change. It would not be possible to obtain complete inhibition, even at greatly elevated concentrations of the cross-reactive antigen. Finally, if the cross-reactive antigen is able to react with all of the antibody population, but with sufficiently different affinities with some than with others, it would be possible to discern the individual binding curves for each population.

Thus, while it may sometimes be possible to identify as cross-reacting a material in a crude mixture where its concentration is not known, the converse is not true. It is not possible to conclude from the shape or slope of the binding curves that 2 molecules are identical.

## RELEVANCE TO ASSAY OF CEA

Results of testing programs in many laboratories indicate, in widely differing disease situations, both malignant and nonmalignant, the presence of material detectable by current CEA assays. From what we have just discussed, it is apparent that similar behavior in a radioimmune assay may result from a diversity of substances. Thus it is of great importance to discern the differences, if any, in the substances detected in these various disease situations.

Here we might cite a case in point. We have found in the urine of normal adults a material inhibiting in our CEA assay. When sufficiently concentrated, this substance is capable of completely inhibiting the assay. We are attempting to isolate this material for characterization. Thus far we have concentrates of a material with a molecular weight in the range of 5–10,000 as determined by Amicon membrane filtration and Sephadex chromatography. While one might hypothesize that this material is a degradation product of CEA, an anabolic intermediate, or a degradation product of a molecule from which CEA derives, the molecular weight alone distinguishes this material from CEA as we normally understand it.

## STRUCTURE AND ORIGIN OF CEA

Ultimately the identity of 2 substances rests on chemical characterization of pure material. Thus

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<sup>6</sup> This does not mean the similar shapes of standard curves indicate antigen identity. This question will be discussed more fully later.

it would seem important to know the structure of CEA and how it arises in the various diseased states, be this by a renewed synthesis of a fetal antigen whose synthesis is normally suppressed completely in adult tissue (2), or by increased synthesis of a normal antigen present in low concentrations in adult tissue (19), or even a completely new molecule.

CEA can exist in at least 2 molecular sizes (14); yet most frequently it seems to be encountered with a molecular weight of about 200,000. This molecule is composed of  $\leq 50\%$  of protein (20, 21). Samples of CEA from various tumors have the same relative amino acid compositions. An examination of the initial N-terminal sequence of a CEA-associated protein has shown that a single sequence is present in the chains unblocked at the N-terminus (22, 23). While it might be difficult to identify CEA-related molecules by their saccharide moieties, the protein sequence should be an exquisite hallmark of CEA-related materials. Nevertheless, we should not forget that the antigenic properties of the molecule may rest in its nonprotein moiety.

For an understanding of the biosynthetic pathways leading to CEA, and possibly to related products, physiologic studies will be necessary. These studies now appear feasible with the finding that a material active in the CEA assay and possessing the same molecular size by gel filtration has been obtained in tissue culture of certain strains of malignant cells (24). Sufficient material is now being purified from tissue culture fluids to permit detailed study of its immunologic relationship to CEA from tumor tissue by double isotope labeling (14), by analyses of amino acid composition, and ultimately by sequencing. Hopefully such studies may shed light on the nature of the malignant transformation by enabling precise studies of the antigenic reversion (1) visualized in the initial studies of Gold and Freedman (2).

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# Carcinoembryonic Antigen in Benign and Malignant Diseases of the Digestive Tract<sup>1, 2, 3</sup>

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**SUMMARY<sup>5</sup>**—The principal clinical application of the carcinoembryonic antigen assay at present is as an aid to the diagnosis of colonic and pancreatic cancers. It has proved to be a better test for widespread metastases than for early cancer. Preliminary observations suggest that the assay may help in the assessment of the adequacy of surgical resection of colonic cancer. It may also help in monitoring the effects of chemotherapy. The present assay methods however are neither specific nor sensitive enough to warrant their use for screening large populations for malignant disease. Further study may uncover immunologic links between benign and malignant diseases, e.g., between a) ulcerative colitis and colon cancer and b) gastric atrophy and gastric cancer.—*Natl Cancer Inst Monogr* 35: 433–439, 1972.

IN 1965 GOLD and Freedman demonstrated the presence of a glycoprotein antigen in human cancer of the colon (4), and in 1969 Thomson *et al.* in the Montreal laboratory described a radioimmunoassay for carcinoembryonic antigen (CEA) in serum (5). Since then, much work has been done

in this laboratory and in other laboratories. The following is an updated summary of some of these studies (1).

## WHAT THE CEA ASSAY CAN DO

### “Recognition” of Cancer of Colon

The initial series from Montreal reported 97% positivity in patients with cancer of the colon (5). This finding was promptly confirmed in this laboratory, where 91% positivity was found (6), and subsequently by LoGerfo *et al.* in New York City (86%) (7) and by Reynoso *et al.* in Buffalo (83%) (8). An enlarged series from this laboratory later reported a fall in positivity to 72% (9) (table 1).

### “Early” Versus “Late” Cancer

In our initial study, to check the assay, we sought patients with “overt” colonic cancers; many of these patients had metastases. Later, we sought patients with “earlier” cancers, and the enlarged

<sup>1</sup> Presented at Conference on Immunology of Carcinogenesis, held by the Oak Ridge National Laboratory at Gatlinburg, Tenn., May 8–11, 1972.

<sup>2</sup> This paper is an updated modification of an article (1) published in the *New England Journal of Medicine*.

<sup>3</sup> Supported by Public Health Service grants CA04486 and CA02090 and contract No. NIH-NCI-E-71-2276 from the National Cancer Institute, by Public Health Service grant TI AM5320 from the National Institute of Arthritis and Metabolic Diseases, and by grant C1-19 from the American Cancer Society, Inc.

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<sup>5</sup> Stillman and Zamcheck (2) and Gold (3) gave a selective summary of papers on alpha-fetoglobulin, carcinoembryonic antigen, tumor glycolipids, and fetosulfoglycoproteins available before 1971.

TABLE 1.—CEA in colonic cancer: variations in positivity from series to series

Series	Method*	Number of patients	Positivity (%)
Montreal (5)	G	36	97
Boston (City Hospital)	G		
Initial series (6)		35	91
Expanded series (9)		60	72
Present series			
Preresection (all stages)†		51	59
Postresection with known tumor recurrence		28	96
New York City (7)	H	101	86
Buffalo (8)	H	33	83
Boston (Lahey Clinic) (14)	G	33	64

\* G: Gold's technique; H: Hansen's technique.  
† Positivity varied from 19% in patients with Dukes' stage A tumors to 100% in those with distant metastases.

series reflects the larger proportion of these. The Gold assay as used in our laboratory as well as the Hansen assay appear to be better tests for widespread metastases than for early cancer. A negative assay does not exclude the diagnosis of early cancer—although it makes highly unlikely the diagnosis of widespread metastatic cancer—and, in patients with known colonic cancer, it suggests a favorable prognosis (10).

Using the Hansen assay, LoGerfo *et al.* (7) indicated that earlier carcinomas of the colon (and also of the breast and prostate) gave negative results for antigen. On the other hand, all 21 of their patients with metastatic breast carcinoma had antigen in their blood. Also using the Hansen assay, Reynoso *et al.* (8) found that, in “nonmetastatic” disease, results in 9 of 10 patients with adenocarcinoma of

TABLE 2.—CEA in colonic cancer: Results on 51 patients tested before tumor resection

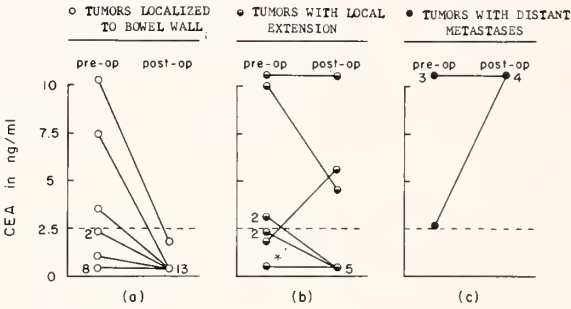
Extent of tumor	Number	CEA positive* (%)	CEA $\geq$ 10 ng/ml (%)
Localized to bowel wall (Dukes' stage A)	16	3 (19)	1 (6)
Extending to pericolic tissues (Dukes' stage B: 3 patients; Dukes' stage C: 14 patients)	17	9 (53)	4 (24)
Metastatic to distant tissues	18	18 (100)	11 (61)

\* CEA level of  $\geq 2.5$  ng/ml in each of duplicate tubes of patients' serum.

the breast were negative, whereas results in 15 of 25 patients with “disseminated” carcinoma were positive. Table 2 shows the correlation, noted by Dhar *et al.* (11) using the Gold assay, between CEA findings preoperatively and extent of tumor observed at laparotomy for resection of colon cancer.

Using the Hansen method, LoGerfo *et al.* also reported a higher frequency of positivity in patients with more advanced colon cancer: Dukes' stage A (38%), Dukes' stage B (60%), Dukes' stage C (75%), and “metastatic cancer” (85%). Microscopic examination of colon carcinomas from patients with and without circulating antigen did not reveal significant differences in mucin production or cellular appearance (12).

Antigens used in the present assays are derived mostly from liver metastases from cancer of the colon. There is no direct evidence to date that this fact contributes to the frequency of positive assays seen in patients with liver metastases.



TEXT-FIGURE 1.—Serum CEA in colonic cancer. Twenty-six patients with both preoperative and postoperative values. Note local extension was accompanied by regional lymph node involvement in all but 1 patient (\*) who had invasion only of the pericolic fat (Dukes' stage B).

Assessment of Adequacy of Surgical Resection

Thomson *et al.* (5) reported 5 patients in whom preoperatively positive assays promptly fell to negative levels after complete resection of colonic cancers. We (9–11) (text-fig. 1), LoGerfo *et al.* (7, 12), and Reynoso *et al.* (8) confirmed this finding. Elevated or rising CEA values post resection were associated with residual or disseminated malignancy and with poor prognosis. Prolonged follow-up observations of more patients are needed to assess further the use of the assay as an index of completeness of tumor excision.

## Recognition of Cancer of Pancreas

Our first 13 patients with cancer of the pancreas gave positive results. Twenty-three (88%) of 26 patients in our expanded series had positive assays (13). Cancer of the pancreas is rarely recognized early. Most of our patients had metastatic cancer, usually involving the liver.

The CEA assay was more frequently positive in patients with cancer of the pancreas than were any other diagnostic tests, including upper-gastrointestinal roentgen series, hypotonic duodenography, celiac arteriography, and percutaneous transhepatic cholangiography. CEA was positive in 3 patients with negative pancreatic biopsies and detected liver metastases twice as often as did liver scan.

LoGerfo *et al.* (7), Reynoso *et al.* (8), and Nugent and Hansen (14) observed positive findings in all instances. Evaluation of early resectable cases is needed.

## Positive Result in Nonentodermal Cancer

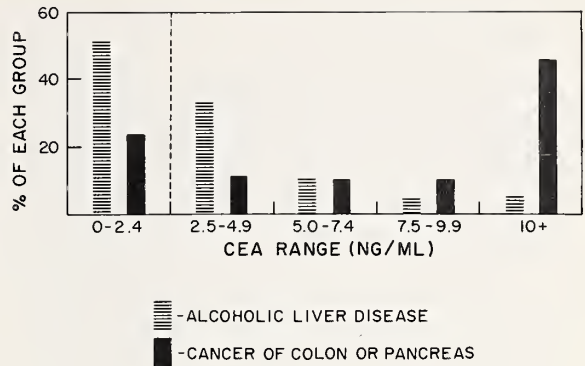
Gold's findings indicated a specificity of the CEA assay for entodermal cancer (5). Neither this laboratory nor other laboratories have confirmed this specificity; positive CEA levels were observed in patients with cancers of the lung, breast, ovary, urinary bladder, and prostate and in patients with neuroblastomas, multiple myelomas, and osteogenic sarcomas.

Reynoso *et al.* observed that plasma levels of CEA decreased after treatment of 6 children with active neuroblastoma, 2 patients with myeloma, and 1 patient with macroglobulinemia of Waldenström (8). No studies have yet been reported of the use of CEA in monitoring the effect of chemotherapy of digestive tract malignancy. The Gold assay was studied in this laboratory with antigens and antisera provided by the Montreal group. The CEA and resulting antisera used for Gold's initial clinical studies may have differed from those in use today by this laboratory and other laboratories (15).

## Positive Results in Benign and Malignant Disorders of the Digestive Tract

### *Alcoholic Cirrhosis and Pancreatitis*

We observed that approximately 50% of selected patients with severe active alcoholic cir-



TEXT-FIGURE 2.—Serum CEA levels in patients with alcoholic liver disease or cancer. Serum CEA levels of 88 patients with alcoholic liver disease (hatched bars) were compared with serum CEA levels of 86 patients with histologically confirmed carcinoma of the colon or pancreas. The number of patients in each range of CEA levels was expressed as a percent of the total group. Levels of CEA  $\geq 2.5$  ng/ml patient serum were positive.

rhosis had positive assays (16, 17). The positivity in these patients did not interfere with the use of the assay in cancer detection, since the patients had characteristic prolonged histories of alcoholism, jaundice, hepatomegaly, spider angiomas, and ascites. The distinction from hepatoma or liver metastases was made by needle biopsy of the liver or by serum alpha-fetoglobulin (or by both). The levels of CEA observed were usually lower in patients with alcoholic liver disease than in those with colonic or pancreatic cancer (text-fig. 2).

Of patients with alcoholic pancreatitis, 50% had positive CEA assays; whereas, of patients with nonalcoholic pancreatitis, none were positive. Positivity was more frequent in patients with underlying liver disease (18).

### *Ulcerative Colitis*

LoGerfo *et al.* (7) reported that 10 of 31 patients with ulcerative colitis, but without cancer, had positive assays. However, using the Gold assay, we found only 1 of 63 patients with a persistently elevated positive assay (9, 19); this patient had colonic cancer. Six other patients had transient CEA elevations without evidence of cancer. Whether this difference was due to differing selection of patients or differing methodology is not known. How useful the assay will be in helping to select the patients for colectomy who have the greatest risk of cancer of the colon remains to be studied.



### *Benign and Malignant Polyps*

LoGerfo *et al.* (7) found positive results in 1 of 29 patients with benign colonic polyps. Only 1 of 15 of our patients with benign polyps had a positive test. This patient had cirrhosis of the liver, and his assay remained positive after resection of the polyp. Of 5 patients with polyps containing foci of adenocarcinoma in our group, only 1 was positive; this patient also had cirrhosis. Using immunofluorescence techniques, Burtin *et al.* (20) identified CEA in all 25 colonic polyps studied with both rabbit anti-CEA antibody and goat anti-CEA antibody (prepared by Gold). Burtin *et al.* acknowledged that Gold did not confirm this finding using the same goat anti-CEA preparation. The intensity of fluorescence varied and, in general, was greater in the well-differentiated polyps than in the undifferentiated ones. CEA was always localized on the apical pole of the glandular cells as observed in carcinomas and in fetal intestine. Burtin *et al.* concluded that "... from an immunological viewpoint polyps behave as cancers."

### *Controls*

None of 40 young persons we selected as healthy controls have had positive results (more than 200 assays). Dr. Gold has informed us that cancer subsequently developed in 40 of 70 elderly patients who had no evidence of cancer at the time of initial positive assay. We have found positive assays in a few elderly patients without identifiable cancers. Many more elderly patients need to be studied. Prolonged follow-up observation will show whether the assay in these patients successfully predicts cancer or whether "old age" itself limits the use of the assay. Many other benign disorders of the digestive tract and of other organs need to be studied.

### **SPECIFICITY OF CEA**

Martin and Martin, in France, detected small amounts of 2 cancer-related antigens in noncancerous colonic mucosa and considered one of them identical to Gold's CEA (27). They concluded that, in terms of these antigens, cancerous and noncancerous digestive tissues differed quantitatively rather than qualitatively. von Kleist and Burtin (22), von Kleist (23), and Kleinman *et al.* (24) did

not identify CEA in normal colonic mucosa. CEA extracted from normal and diseased nonmalignant mucosae needs to be identified physicochemically before the question of whether CEA is present in them is completely answered.

Kupchik and Zamcheck (25) extracted small quantities of glycoprotein, immunochemically identical with CEA, from normal and cirrhotic livers. The concentrations were 1:5,000 to 1:10,000 times less than those extracted from hepatic metastases from colonic cancer. Coligan *et al.* did not find significant amounts of CEA in normal colon or liver tissue extracts (26).

Thus, the tumor specificity of CEA reported by Gold and Freedman (4, 27) is being investigated. The present assays for CEA, like the test for alpha-fetoglobulin, are not diagnostically "specific" for cancer alone.

### **IMPORTANCE OF QUANTITATIVE AMOUNTS OF CEA**

It appears that "malignant" may be differentiated from "nonmalignant" diseases partly by the amount of CEA-like substances found in serum. The present low serum threshold of positivity (2.5 ng/ml is generally used) misses fewer cancers at the price of reporting more "false-positive" assays. By setting a higher threshold (*e.g.*, >5 ng), one may achieve a more reliable diagnosis of positivity but may miss some cancers; unfortunately, at that threshold, many of those cancers missed with the present assays are likely to be early lesions. An IgM anti-CEA antibody was demonstrated in serum of patients with primary digestive system cancer but not in serum of patients with metastatic cancer (28). The identification of anti-CEA antibody in patients bearing primary tumors could be an important adjunct to the CEA radioimmunoassay. This antibody test would permit early diagnosis, and the antigen test would help assess the presence and extent of metastases and adequacy of surgical resection. Collatz *et al.* (29) and LoGerfo *et al.* (30) did not find circulating anti-CEA antibody in patients with digestive tract cancer.

At present, with existing assay methods, the use of a high "threshold of positivity" has some advantages (though there is room for disagreement on this point). Experience has taught pathologists that "false-positive" diagnoses of cancer may lead to disastrous, unwarranted surgery. Established

diagnostic practices should be followed until more experience with the use of these and newer assays has been gained. Although workers in the field may appreciate the limitations of this new approach to diagnosis, practicing physicians must be educated.

## METHODOLOGIC IMPLICATIONS

The methods used for preparation and detection of CEA are highly complex and cannot be used "routinely" as the alpha-fetoglobulin test is used. The antigen needed for the assay is extracted usually from metastases of colonic cancer to the liver (in surprisingly limited supply), very large amounts of which yield only small amounts of the antigenic glycoproteins. This material must be used for preparation of antibody (usually by immunization of a goat or rabbit), for labeling with radioactive iodine for use in the radioimmunoassay, and for use as a reference standard. The Montreal (Gold's) assay (5) analyzes serum, and the Hansen assay (7, 31, 32) analyzes plasma. The antibody used and the tumor-associated antigen (TAA), recognized in the Hansen assay, may differ from those in the Gold assay. LoGerfo *et al.* stated that "tumor-associated antigen" has an ion-sensitive site, "TAA," found in normal colon and lung and also on CEA. They hypothesized that CEA differed from TAA in being specific for entodermally derived tumors (33, 34). In this report we have used the term CEA to encompass findings with both systems, but we realize that future work may distinguish between the two.

A comparative study of both assays, using the respective antisera, performed simultaneously on the same patients' blood specimens, was done by Sorokin *et al.* in this laboratory (35, 36). Apparent "differences" between the 2 assays were primarily quantitative. In the low ranges of antigenemia, values obtained using the Hansen technique were slightly higher than those obtained on the same blood with the Gold technique. The Gold assay tended to read higher than the Hansen assay in the group with alcoholic liver disease, when values (Gold) were  $>4$  ng/ml.

The lowest frequency of agreement between assays was in those groups with low levels of antigen, such as postoperative colon carcinoma and inflammatory bowel diseases. This probably reflects

variations inherent in the assays as currently performed.

In only 1% of the 567 comparisons was the level of antigenemia "positive" in one assay and "undetectable" ( $<1$  ng) in the other. Small variations ( $\pm 1$  ng/ml) around 2.5 ng/ml accounted for 83% of the apparent noncorrelations between "positive" and "negative" values. Further study is needed to define "positivity."

Other assays (37, 38), including the Todd system, are being studied for possible clinical use. Antigens must be extracted from normal and malignant tissues of all organs and compared. Assay methods must be developed to exploit differences in type or amount of antigen.

## DISCUSSION

Thus, the CEA studies to date support the promise of a clinically useful test for the detection and diagnosis of cancer of the digestive tract that will also help assess adequacy of surgical resection and the presence and extent of metastases. Quantitation of the assay (or an improved one) will permit comparison with tumor histology. A new quantitative assessment of "malignancy" will emerge from the combined immunochemical and morphologic experience. The use of this new method may permit better evaluation of premalignant states, such as ulcerative colitis (39) and polyps.

When the CEA assay or its successor becomes perfected and reagents are standardized, immunochemical diagnosis will be an important adjunct to (but not a substitute for) experienced clinical judgment and complete X-ray and laboratory study. Whether it will do so early enough to improve prognosis remains to be determined. The performance and interpretation of such assays will require the participation of pathologists and others experienced in the practical diagnosis of malignant tumors and aware of the risks to the patient of misuse. Much work remains to be done before widespread "screening" is feasible or warranted.

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## DISCUSSION

**P. Burtin:** We intend now to have a discussion of all the findings on carcino-fetal or carcinoembryonic antigens. I suggest we begin by first discussing alpha-fetoprotein and then discussing carcinoembryonic antigen (CEA) in tissues and plasma. Are there comments on alpha-fetoproteins?

**H. T. Wepsic:** We have been assaying alpha-fetoprotein levels in patients in San Diego, and recently we had a patient who had a "teratoblastoma," a poorly differentiated tumor on the mediastinum. We feel in these particular cases we will be seeing alpha-fetoproteins in these patients.

The other comment I want to make is that hepatomas often occur in postnecrotic cirrhosis. There is a series out from the Virginia Medical School showing this striking development, and there is also a correlation in some studies with Australia antigen. So we will have some very interesting results over the next few years from studies of where and why hepatomas develop.

Dr. Alpert, would you comment on the counter-current diffusion technique? Specifically, would you comment on nonspecificity of any problems that one might run into? There are a number of us that would like to know what these problems may be.

**E. Alpert:** I want to add a note of caution concerning interpretation of precipitin lines. Not every precipitin line is an immune precipitate. To illustrate this point, figure 1A shows an electroimmunodiffusion plate, using monospecific anti-CEA antisera against the sera from a number of patients with carcinoma of the colon, as well as controls. A number of precipitin lines can be seen between the antisera and some of the colony cancer patients.

Figure 1B shows the identical group of sera from patients with carcinoma of the colon and normal patients, run against unimmunized rabbit serum. Precipitin lines are clearly visible, which are obviously not immune precipitates but nonspecific rabbit-human protein interactions. These do not occur when saline is substituted for rabbit sera.

So I would like to strike a note of caution and re-emphasize that not every precipitin line in agar gel is an immune precipitate.

**M. M. Sigel:** Dr. Alpert, simply because your rabbits were not immunized deliberately does not mean that you were not seeing antibody-precipitating reactions. After all, pregnant animals may have antibody to CEA.

**Alpert:** If rabbits, taken at random like that, have antibodies to CEA, then it would raise a lot of questions about a lot of rabbit immune systems that have been in use for a long time.

**A. Chernoff:** For the sake of completeness and because it is of some interest, it ought to be pointed out that fetal hemoglobin was probably the first CEA studied in detail. In the early 1950's, it was shown to be elevated in a number of tumors at levels far in excess of the levels we are seeing here described for CEA. It doesn't bear an alpha-fetoprotein *per se*, but it is a factor that ought to be brought out.

**Burtin:** Now I think we should go on to the discussion of CEA. First, are there comments on the presence of CEA in either cancer or noncancerous tissues?

**Wepsic:** Dr. Burtin, did you ever absorb your fluorescent antibody with a colon tumor positive for CEA and then, after exhaustive absorptions with that material, react what remained of your fluorescent antisera with normal tissue?

**Burtin:** Of course. The use of absorbed antisera is a necessary control. We compared absorbed antiserum, antisera absorbed with homologous antigen, to unabsorbed antiserum. All the patterns which I showed were controlled by the use of absorbed antiserum, and of course they were judged as valid only if the absorbed antiserum did not give a positive reaction.

**A. S. Garrett:** Dr. Zamcheck, in the healthy individuals who have positive CEA tests, are the clinical histories good enough, for example, to find out other things that they may have in common, such as smoking?

**N. Zamcheck:** We do have additional data on healthy subjects. In one group of 823 supposedly healthy subjects, 11 individuals had CEA titers over 5 ng/ml. In this over-all group, 39 individuals were heavy smokers, and 9 of the subjects with titers over 5 ng/ml were in this heavy smoker group. So this may or may not give us a lead on something.

I would also like to point out, as many of you know, in routine autopsies at many hospitals in the world there is a significant percentage of carcinomas found in individuals; carcinomas were not suspected. It is problematical how much can be accounted for in these so-called healthy subjects by these occult carcinomas. These patients are subjects included in these studies and, as far as we know, have not had extensive work-ups since these titers were first detected. They may have proceeded later, but we don't have any data on them.

**Burtin:** May I suggest we direct this discussion to a precise point, that is, the comparison between different methods of detection of CEA. As you know, there are different methods for radioimmunoassay: that of Dr. Hanson, Dr. Gold, and that of Dr. Todd. I don't know if these methods have been compared, and if they gave the same results for the same sera.

**C. W. Todd:** I'm not sure I'm the one who should comment, because we haven't tried to do our clinical study. We've been more interested in developing the test and studying the antigen. We've looked at enough clinical material, however, to make sure we are in contact with reality.

We have helped the Chester Beatty Research Institute in London set up the assay. This institute has tested many sera which have also been fed into the Hoffman-LaRoche assays. The conclusions which I have gotten from Dr. Peter Alexander at the Chester Beatty Research Institute are as follows: 1) The 2 assays correlate; and 2) there are a few examples in which the results are high by the Chester Beatty Research Institute assay using our technique and low by the Hoffman-LaRoche assay. At the same time, there are a few that are high by the Hoffman-LaRoche assay and low by our assay. The percentage, or the number in each of these 2 categories, is about equal. So you can't



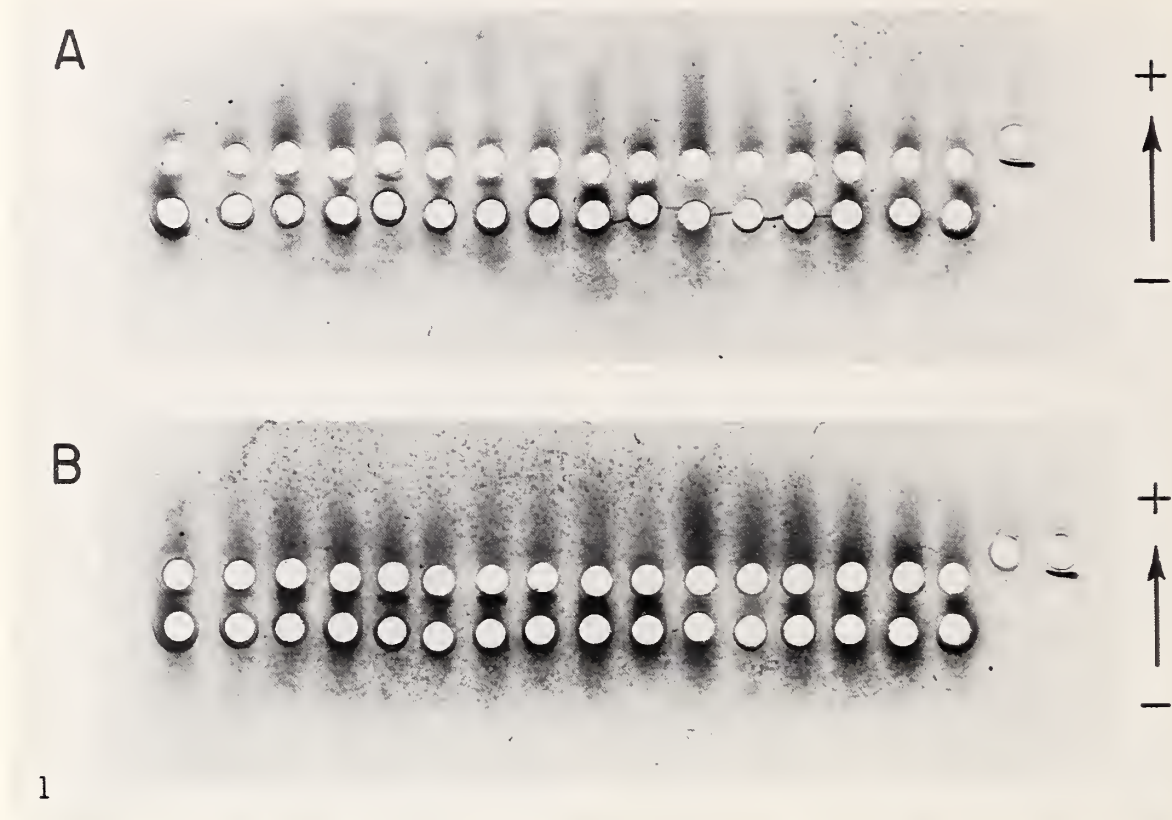


FIGURE 1.—Counterimmunoelectrophoresis in 0.05 M agarose, pH 8.6. Bottom row of 1A and 1B contains identical patient sera migrating anodally. In A, top row contains cathodally migrating rabbit anti-CEA monospecific antiserum. In B, top row contains immunized rabbit sera.

say that one tends to be high while the other tends to be low. Exactly what these differences in titers mean is uncertain at this time.

The Hoffman-LaRoche assay starts out first with a perchloric acid precipitation. In our hands there's always a certain amount of CEA activity lost during these manipulations, and we start out directly with the serum.

The experience at the Chester Beatty Research Institute is that there seems to be something of the order of about 7 ng/ml as a blank, if you compare normal serum to a standard made on a buffered saline curve. Just why their blank is higher than ours I don't know. But at any rate, when you go down to very low levels, then you face the question of how you are going to treat the low levels in comparing the two. The fact of the matter is that normals seem to be a little higher in our assay than they do when you precede this by an extraction technique.

**Zamcheck:** We compared about 500 assays, doing both the Hanson or Hoffman-LaRoche assay, and the Gold assay in our laboratory. We also sent a portion of the specimens to Dr. Hanson at Hoffman-LaRoche for comparison

to the Gold assay. In about 85% of the cases, the assays were identical for all clinical purposes. Discrepancies always occurred at the low antigen level. The Hanson assay tended to read higher than the Gold assay for reasons that Dr. Todd has already enunciated. Also, I agree that the blanks that one uses are quite crucial. If you use a blank as we did, from a hospital population, we found that it had higher levels of antigen than a standard blank as used by Hoffman-LaRoche, so that this may account for some of the discrepancy.

**R. P. Lange:** I would like to point out that there is an alternative method for assays of CEA. We have approached this problem at the University of Tennessee Memorial Research Center by using a hemagglutinin-inhibition assay rather than a radioimmune assay. I think that our results compare quite favorably with the ones that have been presented. For instance, in cases of known carcinoma of the colon, we have had 28 of 30 that have been positive. In our control population of some 373 patients tested—and I should like to point out that these controls included all age groups without malignant disease—40 positives were ob-

tained, about 10%. But I would like to re-emphasize that this was a controlled population of all ages. Also, we had one positive result in a 2-mm polyp found to be malignant.

**N. L. Warner:** We have also developed an assay very similar to that of Dr. Todd. We used double antibody and also compared the same sera with the standard extraction. In both cases, the only difference from the classical method was that we were working only with 25  $\mu$ l of whole serum. With this direct serum test, our results were very similar to those presented earlier.

The critical distinction comes, however, when one compares the 2 assays the following way: Whereas in cases of colon carcinoma, lung carcinoma, stomach carcinoma, and alcoholic cirrhosis, positives are obtained by both direct serum and extraction tests, in other nonmalignant gastrointestinal disorders, we find something like 30% positive by direct serum, but no positives on perchloric-acid-extraction tests.

**Burtin:** This is a very important point.

**E. Klein:** Mr. Chairman, I've been asked by the Roswell Park group to say that their data correlate very well with those presented by Dr. Zamcheck, and that the immediate

clinical use that they find is in monitoring their patients postoperatively during chemotherapy to determine what is happening to the cancer when they cannot define this clinically.

**Burtin:** Dr. Coligan, have you more precise data to submit regarding this point?

**J. E. Coligan:** Not really. We let the other groups do the clinical studies. As far as making a point, I think that something that should be kept in mind here is what Dr. Todd tried to point out: Nobody knows for sure if he has a pure antigen or a pure antibody. Until you get this defined chemically pure and are sure that you have a pure antibody, you are not going to be sure what you're actually assaying.

**Burtin:** It's time for us to draw a conclusion. As you can see, there is much work already done on this point of radioimmunoassay. I think much is yet to be done in this entire area, and I hope that in the following months or years, we can know much more regarding purification and characterization of the antigens, as well as specificity of the antibody, in this over-all problem of detection and diagnosis by use of carcinoembryonic antigens.





# Summary of the Conference on Immunology of Carcinogenesis<sup>1</sup>

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I know I speak for all the participants in thanking those who worked to make this third conference of a series another successful review of an important topic in cancer. The first in the series was the Conference on Inhalation Carcinogenesis, held in October, 1969 (1). The second was the Conference on Morphology of Respiratory Carcinogenesis, held in May, 1970 (2). We are especially grateful to the Conference Committee who organized the meeting: Drs. Hanna, Rapp, Borsos, and Rice. From the excellent scope of the program, however, the title of the Conference should have perhaps been "Immunology and Cancer."

After welcoming remarks by Dr. Weinberg, who mentioned the major revolution in the biologic sciences beginning with Pasteur, Dr. Hanna presented to us the 3 challenges to immunology as seen by Dr. Rauscher:

1. What can immunology do for the prospective cancer patient?
2. What alternatives can immunology provide for the patient with disseminated cancer?
3. What kinds of tumors can be affected beneficially by immunology?

Also, if we cannot deal with these questions satisfactorily now, what needs doing to do so?

## SESSION 1

Dr. Borsos opened the presentations and set part of the tone for the meeting with his statement that: "All immunology is aimed at finding out what the differences are between the tumor cell and the normal cell and measuring the differences." He reminded us that an antigen is an antigen only when a host responds to it. He averred that prevention of human cancer is the primary goal. Therapy is seen as the second major goal, and he gave emphasis to models that should be developed, including those aimed at helping the host reject tumor.

Within this session was a sound critical review of current *methodologies* for detection of antigens, with emphasis on preneoplastic and neoplastic tissues. While some participants in the Conference may not have felt the need for this review of methodologies, I am sure many of us benefited from specific points with which we were not familiar. Moreover, it should be remembered that the proceedings of this Conference will be published, and this review will be of great benefit to students and those who may wish to enter the field.

Much has been written on the influences of methodology and techniques on the conceptualization of a field (3-8), and we must be careful not to let the techniques unduly distort, especially when laboratory methodologies may make it more

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difficult to discern the picture *in man*. The papers on methodology also emphasized difficulties sometimes present in interpretation of the meaning of the results after they are in hand. Methodologies are subtly very important in molding our understanding of our data. One might recall an old story about early oceanographers who set out to study life in the ocean. With their nets of 2-inch-square mesh, they returned with the marvelous discovery that there was nothing smaller in the ocean than objects of about 2-inch size. I suspect that some of our techniques are producing analogous conclusions within us, but they are so sophisticated, we don't notice them!

Drs. Levy and Winn covered antibody-mediated tumor immunity. Drs. Nordin and Bartlett covered cell-mediated tumor immunity. Advantages and disadvantages of the various tests were discussed, and these features can be more clearly drawn than can the issue of their relevance to cancer. There were a number of highlights from this session, which, despite their being tied to methodologies, were of general significance beyond methodology. They were:

1. More studies are needed with both blocking and cytotoxic antibodies on tumor cell surfaces.

I think we can easily agree with that statement. There are fascinating leads, but many complexities are with us, and we will need to work out many more quantitative studies with correlations ultimately for man before we can make sense out of some of the results. Let me digress a moment and say that different fields have different stages of maturity. The viral oncology area, for example, is somewhat more mature than the cancer immunology area, and things look a little clearer, perhaps, in viral oncology (9). But I sometimes wonder if this is not a danger. I'm beginning to wonder if we are not producing a lot of laboratory curiosities and artefacts by being so quantitatively circumscribed in some laboratory procedures which finally end up with little or no relationship to man. It may be a healthier situation with immunology, because it is still more closely related to man and the complexities and more immature state-of-the-art are probably insuring broader coverage of relevant areas.

2. Differences in lysis of red blood cells and nucleated cells were noted.

In other words, there is considerable question whether studies with red blood cells really have much bearing on studies with cancer cells.

3. Some tests such as assay for carcinoembryonic antigens (CEA) are already useful in diagnosis of some forms of cancer, and it can be anticipated that they will become more so in the future; some are beginning to be useful in following courses of treatment.

A good demonstration of this value was presented in the latter part of the Conference, particularly in regard to following courses of treatment. It is obvious that the CEA test as it is now run is not going to provide the kind of diagnostic and detection tests that some hoped for. You may recall that the National Cancer Institute evaluated about a decade ago some hundred laboratory tests claimed to be of value in diagnosis and detection; the Pap smear was the only one that held up. These tests were chemical, immunologic, and cytologic, for the most part. I hope we can do better in the current efforts, but this particular area of cancer research is especially difficult, because cancer most frequently is initiated with one or a few cells in the milieu of billions of noncancer cells, and that feature alone makes it difficult to pick up cancer early. Yet, the possibility exists that cancer, from some points of view, is a systemic disease and may be detectable by a more general set of metabolic characteristics. Indeed, with some kinds of cancers, such detection can already be made. Newer, automated techniques offer new possibilities for assaying large numbers of samples if the logistics can be efficiently handled (10).

The other great difficulty is borne out in this Conference: the biologic variability among tumors and among individuals with the variation of response of their immune systems. One

usually finds overlaps of bell-shaped curves, whatever characteristics are being evaluated, and it is with the problem of overlap of the curves in the middle ranges that we always have difficulties. Ordinarily an arbitrary line, *e.g.*, 2.5 ng of antibody nitrogen per ml of serum, is drawn to try to optimize this balance and minimize the false positives and negatives, but it is a tough area, so I encourage those of you in the field to keep at it, because it is of great importance.

4. Newer microtechniques have become very sophisticated and utilize very small amounts of materials.

This is a very important advance for work with patients. At times I worry about bleeding some patients excessively because we sometimes take so many samples for clinical investigation. The development of microtechniques, while seemingly a small point, is extremely important for progress in clinical research.

5. The major difficulty with *in vitro* tests is getting at biologic activities expressed in the *whole* individual.

We try to overcome that, of course, by correlating the results back and forth between *in vitro* and *in vivo* studies.

6. Very few examples are known where vascularized, established tumors have been rejected by means of humoral antibodies.

We saw some examples of modification of established tumors in the latter part of the Conference. I am not sure whether we can conclude that this was accomplished with *humoral* antibodies. The more fruitful path of research is probably not in this direction, but in the direction of *cell-mediated* activities.

7. We were reminded of the danger of tumor enhancement.

Enhancement or inhibition can occur in the same genotype, though more frequently there are differences among different histologic types, and there may be differences in the same tumor at various times, *i.e.*, at different stages of growth.

8. Serologic tests are not reliable guides for determining enhancement.

9. Enhancement or inhibition depends on complicated issues, such as concentrations, specificities, biologic and chemical properties of antibodies and half-lives, cell differences, etc.

This is unfortunately the case, and it means that we will have several years of work ahead of us to try to move toward greater quantitation and extensive additional correlations to unravel these complexities.

10. Some reactions against tumors are with the vascular tissue, not tumor cells *per se*, and the vascular tissue can be, and frequently is, derived from the host in transplantation studies.

11. Variations among tumor cells give variations of effects among cells.

It is difficult to kill all the cells; if all the cells are not killed, then usually the tumor will grow again. This can be very discouraging to us, but we have lived with biologic variations of cancers and cancer subjects for a long time. We will probably not always obtain a 100% kill, but we can improve.

12. *In vitro* cell-mediated methods were developed primarily for studies on transplantation or histocompatibility, but the key issues in cancer relate to tumor specificity of antigens.



This problem is being overcome rapidly with all the work going on, and the older tests are being modified with greater focusing on tumor specificity in terms of the antigens.

13. Tumor-specific antigens exist and react with lymphocytes as both soluble and cell-surface antigens, but more work is especially needed in isolation and purification of these antigens; better quantitation is required, and additional correlations must be made between *in vitro* and *in vivo* systems, particularly in man, if immunology is to be important for diagnosis and treatment (and also in eventual prevention).

We must always think of man, and I again emphasize the necessity of the pairing up of the clinical investigators with the laboratory investigators. I'm hopeful that this blending will increase. It is growing.

14. *In vivo* cell-mediated models can be of importance in 3 areas: *a*) Transplantation methods can provide models of *in situ* primary tumors (there may be some disagreements on this conclusion); *b*) experimental carcinogenesis studies can serve as models of naturally occurring tumors (there is probably less disagreement here); and *c*) animal studies are models of human neoplastic disease and aid in studying processes.

15. Technologic goals were mentioned: *a*) standardization of reagents; *b*) greater sensitivity; *c*) distinguishing between cytotoxicity and cytostasis; *d*) study of individual components; and *e*) improved quantitation of antigen and antibody amounts and other determinations.

The need for standardization of reagents is an area that is boring to many people. It is difficult to define and reach agreement on how reagents should be standardized, and yet it is a crucial area. Difficulties with standardization of reagents led to several early problems in the CEA tests, for example, but they seem to be being ironed out now. This area also brings to mind the necessity of industrial participation in those studies requiring large-scale quantities. In this sphere, industry can often produce materials better than can be done in any laboratory, provided the specifications and the assays used for those materials are those used in a good laboratory and are maintained through the contractual monitoring arrangements with the industrial firms. We have learned from some of our experiences in the programmed areas of the National Cancer Institute that this can be done if the proper "specs" are developed, and the proper quality controls are run. It is the only way to provide sufficient quantities of reagents to deal with certain kinds of questions. In many of these assays, the number of steps involved in the mechanisms are probably several, and full insight requires the breaking down of the steps into individual components. Complement, of course, is an area that has illustrated the value of this type of intensive study, thus providing a great deal more insight into complement, complement fixation, and a number of other reactions.

16. Immunity may not be the same at all sites in the body.

17. Coating of cells with different antibodies produces different effects with the same types of cells.

18. Metastases grow at different rates compared with the primary tumors, and factors other than immunologic ones affect growth and other characteristics of tumors.

We tend to focus in such a specialized way on the subject at hand that we may attribute many of the effects to factors solely within the defined area of study and forget about other characteristics outside.

19. To improve probability that results will be relevant for man, better models are needed of primary autochthonous tumors.

20. *In vitro* studies in man should be made at different stages of clinical disease.

## SESSION 2

The second session covered antigens in preneoplastic tissues during tumorigenesis. Highlights include the following:

1. Immune surveillance was depicted as inadequate.

There was some agreement that perhaps the concept of immune surveillance, so provocative in the last few years, may have outlived its earlier usefulness; this idea is worthy of additional thought.

2. It was proposed that a key function of the immune system is to regulate the expression of components on the cell surface and thereby maintain cell types at an appropriate level of differentiation.

This idea, of course, implies a feedback control and a balance of immunologic (and other) factors that maintain differentiation. The evidence for this idea is certainly incomplete, but it is another direction worthy of additional thought and experiments.

3. In the very interesting striped mice produced by the Mintz technique, which in effect produces an individual with 4 parents, 3 distinct types of tumors were produced in a single tissue: alveolar, ductal, and epidermal mammary tumors; premalignant tissues were found to contain antigens that cross-react with tumor antigens.

We had some argument—as we often do—about what premalignancy is. In the epidemiology field, it is usually those lesions that later become cancers with a high statistical frequency, *e.g.*, leukoplakia. The pathologists may have a different definition by emphasizing morphologic appearances and placing the decisions within a large body of knowledge on behavior of tissues based on long experience. But whatever definition we might settle on, there is no doubt about certain events occurring before the tissues are of such an appearance that most pathologists would agree that cancer is present. From the Prehn and DeOme schools, we have available important precancerous lesions for study. When chemical carcinogens are applied systematically, obviously we know that some of the cells exposed to the carcinogen are going to produce cancers later, and this system provides a means for getting at the complex issues under the general label of premalignant tissues. Promoting effects (*e.g.*, with croton oil) in mouse skin-painting experiments, of course, provide an additional probe on mechanisms of action.

4. In human breast cancer, one can find histiocytes in draining lymph nodes and lymphocyte invasion in the breast similar in extent in both *in situ* and invasive areas, and prognosis is better with lymphocytic infiltration; such infiltration is particularly frequent in *in situ* breast carcinoma, suggesting activity of tumor antigens very early, if not in the preneoplastic state.

5. The serum of a rat with mouse sarcoma virus (MSV)-2 transplanted tumor inhibits reverse transcriptase activity, but not activities of 2 normal cell polymerases; antibodies were prepared against purified mouse C-type viral enzyme, and inhibition was found when various templates were employed, thus suggesting an immune reaction against antigens of an enzyme necessary for the induction of virus-induced tumors.

6. Three recent isolates of primate C-type viruses are not inhibited by antibodies against either murine or feline C-type enzymes (important in the search for human cancer-inducing viruses).

The RD-114 isolate may indeed be important in human cancer induction, since not only was it isolated from man, but also it does not seem to be related to any of the other type-C viruses known to produce tumors in animal systems even though it is morphologically similar as seen with the electron microscope.

Dr. Weiss summarized this session with a long series of provocative questions (*q.v.*) which will guide many investigators in selecting future work. These questions represent the major part of cancer immunology currently and over the next several years (11-17), and we can all look forward to an exciting time as the data become available aimed at answering the thoughtful questions posed by Dr. Weiss. I invite everyone to review carefully these highly significant questions in Dr. Weiss's paper. As the efforts in these immunology areas become more closely correlated with chemical carcinogenesis results, wholly new opportunities will become available for prevention of cancers. Potentials also exist for identification of individuals who have higher risk to cancers as understanding grows and some of the questions are answered.

### SESSION 3

The third session was concerned with antigens in neoplastic tissues. Dr. Girardi, on opening the session, raised important questions on this subject, largely from a molecular biology perspective. Here again, I invite everyone to consider the important ramifications of Dr. Girardi's paper for their impact on future conceptualizations and experimentation.

1. Antigenic expression in neoplastic cells results from transcription and translation of nucleic acid sequences different from those ordinarily expressed in normal cells; it may result from variations of secondary structure, alterations of cell and metabolic kinetics, or shifts in the profile of proteins already formed, and these changes may have controls at the genetic level.

2. New, expressed specificities may be "retrogenic," as suggested by similarities shared by fetal and neoplastic tissues and components (18) or may be induced by foreign nucleic acids introduced into cells; much work over the next few years will be directed to determining the extent and degree of generality of these mechanisms.

3. Chemical induction of tumors may involve frameshift mutations, activation of repressed fetal antigens or of integrated viral genomes, and enzymatic excision in host cell nucleic acid; these changes may or may not be important in inducing immunity or cancers.

4. Host genetic control may be expressed in several ways, including immunity induced against disease and susceptibility to infection, or change, at the cellular level; working out the interplay of host genetics and immune expression will entail many experiments over the next few years.

5. Transformed or tumor-derived cells removed from the host can serve as immunizing inocula after inactivation to reduce their oncogenicity; extraction,



purification, and characterization of tumor cell components will allow movement toward needed improved quantitative immunobiology.

Papers presented in this session made contributions in all these areas. Highlights include the following:

1. Development of local sarcomas produced by 3-methylcholanthrene (MCA) in strain I mice was increased in mice treated with rabbit antimouse lymphocyte serum but not in C3Hf mice.
2. Two types of resistance were shown toward Friend leukemia virus: one that could be overcome by rabbit antimouse lymphocyte serum and one that was insensitive to it.
3. No differences in antigenicity were found among tumors arising in normal, normal rabbit serum-treated, and rabbit antimouse lymphocyte serum-treated I or C3Hf mice.
4. Immunosuppression is not required for cancer induction; no correlations were found between carcinogenicity and assays for IgG, agglutination, and hemolysis.
5. The number of MCA-induced tumors in skin grafts was less when the recipients were chosen from resistant strains.
6. Factors other than immunologic ones are involved, *e.g.*, penetrability of chemicals and viruses, stickiness of virus on cells, etc.
7. In cloning experiments with MCA applied in diffusion chambers to progeny of a single cell, the antigens of tumors induced were still individually distinct even though they all had recently originated from a single cell.
8. MCA-induced, transplantable papillomas and carcinomas of rat urinary bladder (and carcinomas of mouse bladder) provide models for study of immune reactions to common antigens of the tissue-specific type (neuroblastomas, melanomas, colonic carcinomas, breast carcinomas, ovarian carcinomas, sarcomas, and bladder carcinomas); lymphocytes from rats with bladder carcinomas or papillomas were cytotoxic to cultivated carcinoma and papilloma cells in colony inhibition assays (similar results were found in mice); blocking antibodies occurred with carcinomas and papillomas; when sarcomas were induced, cross-reactivity with carcinomas did not occur.
9. New opportunities for better quantitation are available with 2 soluble antigens from diethylnitrosamine-induced tumors in strain-2 guinea pigs, purified severalfold with protein fractionation techniques (mol wt  $\simeq$  70,000–150,000); fractionation was monitored by determination of the capacity to cause a delayed-hypersensitivity reaction in guinea pigs immunized intradermally with fractions derived from 2 antigenically distinct tumor cell lines; studies are under way to learn if results in animals immunized by other routes of administration will yield results comparable to results from these intradermal studies.

Professor Baldwin, in summarizing this session, discussed several aspects of tumors: Not all tumors have detectable antigens; some have very strongly acting

antigens; some have very weakly acting antigens (discussion emphasized that variations in results are partly due to differences in assay systems). Specificities of tumor antigens vary. Immunosuppression is not correlated with carcinogenicity. The need for more study of cell surfaces was cited: Cross-reactions occur between embryonic cells and soluble cytoplasmic proteins, but not always with tumor cell membranes. The requisite methodologies are improving, and better characterization of tumor antigens is now possible, especially with development of solubilization capabilities. However, new model systems are needed, since most chemical carcinogenesis models are developed with polycyclic hydrocarbons. Moreover, results from tumor immunologic studies must be related more fully to tumor rejection, and additional efforts are needed to correlate *in vivo* and *in vitro* results. Combined virologic and chemical carcinogenesis approaches, especially *in vitro* transformation systems with virologically characterized cell lines and chemical agents (including extracts from city smog), offer new possibilities for assays and model systems (19).

#### SESSION 4

This session was on modification of immunity and carcinogenesis. Dr. Stjernswärd opened the session with a discussion of the literature on immune surveillance and evidence indicating the existence of tumor-limiting host factors (*q.v.*). Several lines of evidence were presented which allow the reasonable conclusion that immune phenomena effectively modify the course of tumor pathogenesis. Confusion still remains as to extent, generality, whether stimulation or inhibition is preferable, timing, and interrelationships, but one can be optimistic that practical usefulness lies down the road as the gaining of more data permits a greater understanding of these complex systems. Very likely, interrelationships involve feedback loops which can be understood and exploited for prevention, detection, and diagnosis, and effective therapy only after most of the total system is seen. Which activities need to be enhanced and which inhibited and in which systems still remain largely to be worked out, and Dr. Stjernswärd wisely cautioned us against potential dangers of enhancement and creating laboratory artefacts useless or misleading for man. The search for correlations between laboratory and clinical (and epidemiologic) data must be continual.

Highlights of this session include the following:

1. A methanol extraction residue (MER) of phenol-killed, acetone-washed, attenuated tubercle bacilli (BCG), which in itself offers no special advantages over other agents stimulatory to immune systems, was selected for intensive study of its capability to modulate immunologic responsiveness to several test antigens; MER reduced incidence (and delayed time of appearance) of leukemia following infection with P127 virus and produced slower growth in chemically induced and transplanted tumors in some systems; treatment with MER in conjunction with radiotherapy and chemotherapy was synergistically effective; in several model systems, *cellular* antibodies were produced if the antigen was weak and the MER dose was low, while *humoral* antibodies were produced if the antigen was strong and the MER dose was high.

2. Skin painting of Syrian hamsters with 7,12-dimethylbenz[*a*]anthracene was used in conjunction with immunologic techniques to determine the relationship of regional and systemic immunity to skin tumor development; a transient suppression of systemic humoral immunity occurred with a proximate, more lasting suppression

of homograft immunity; papillomas first appeared when the suppression of humoral immunity was maximal, and malignant transformation commenced at the beginning of the transitional period between recovery of humoral immunity and depression of regional cellular immunity; in lymphadenectomized and splenectomized animals, papilloma latency and regression were reduced; for precise interpretation, these interesting correlations will require further work, especially with respect to time relationships.

3. Data were reviewed supporting the concept that chronic immunosuppression with persistent antigenic stimulation increases the risk for developing malignancy of immunocompetent tissues, and additional data in 2 animal model systems were presented.

4. In experiments with Wistar rats given subcutaneous injections of 3-hydroxy-xanthine, the relation was studied between host immunity, as modified by aging, and susceptibility to chemical oncogenesis; neither aging nor the carcinogen influenced antibody formation or rejection of tumor graft; tumors originating in older animals grew faster than those originating in younger animals; under the conditions of this study, no clear relationship was seen between the level of immunologic response early in the study and eventual susceptibility to chemical tumor induction.

5. A new research tool of potential importance was described: spontaneous tumor regression in about 12% of Swiss or NZW mice with stem cell or poorly differentiated lymphocytic lymphomas of thymic and nonthymic origin induced by 1-ethyl-1-nitrosourea. Progressive aplastic anemia usually accompanied the regression.

Dr. Haran-Ghera, in her summary remarks for this session, noted that several of the speakers had presented results indicating correlations between immunosuppression and tumor induction, but also noted that few studies have been made in which each animal was followed as to the immune state with later determination of tumor incidence. She presented data from several studies using several techniques which did not indicate positive correlations. During the discussion, it was noted that most patients with Hodgkin's disease do not have a history of infections (though epidemiologic data, especially from a virologic standpoint, are inadequate). With reference to data on cancer incidence in kidney transplant patients, it was noted that other patients with kidney disease (*e.g.*, those on dialysis machines) might constitute more proper controls than the general population. To date, it appears that the concept of altered immunity affecting tumorigenesis is valid, but that much more work will be needed to determine under what conditions the phenomenon occurs and can be exploited.

## SESSION 5

Session 5 was on interaction of humoral and cellular mechanisms in tumor immunity. Dr. K. E. Hellström opened the session by noting that it is possible, with appropriate techniques, to find group antigens in chemically induced tumors in addition to virus-induced tumors and to find unique antigens in particular virus-associated tumors in some cases. He also emphasized the significance of the ability of serum antibodies from cancer patients to kill cancer cells *in vitro* (in the presence of



complement) and introduced the topic of blocking antibodies. Major questions exist on the roles of macrophages and of B-lymphocytes and T-lymphocytes and on the relationships of *in vitro* and *in vivo* data.

Highlights of this session include:

1. Thymus-derived lymphocytes can participate in some *in vitro* lymphocyte-mediated cytotoxicity reactions, but this is not the general rule; humoral antibodies can block this cytotoxicity and can also induce cytotoxicity of normal lymphocytes (mediated by IgG antibodies with thymus-independent lymphocytes); patients with urinary bladder carcinoma have specific cytotoxic lymphocytes and tumor-specific humoral antibodies, and the lymphocyte cytotoxicity can be inhibited by extracts of tumor tissue and with tumor-specific glycoproteins; however, the specific cytotoxicity *in vitro* of such a patient's blood lymphocytes is reduced when tumor growth is more extensive, in contrast to reports on other human tumors.

2. Blocking activity is associated with antitumor antibodies, most likely in the form of antigen-antibody complexes; these antibodies are adsorbed on tumor cells *in vivo* and can be eluted at low pH; passive transfer of blocking sera or eluates to animals previously given a tumor isograft leads to enhanced tumor growth; blocking activity can be counteracted by splenectomy or inoculation with certain sera containing antitumor antibodies plus "unblocking antibodies"; when eradication of blocking can be maintained for some time, tumor growth is inhibited, a finding of possible practical significance if the effect can be enhanced with sufficient generality.

3. Results were presented on stimulation of DNA synthesis by lymphocytes from peripheral blood or local lymph nodes from patients with active cancer when the lymphocytes were mixed *in vitro* with autochthonous malignant and nonmalignant fresh biopsy cells in which the DNA synthesis had been blocked (MLTI test); the circulating lymphocytes were more often stimulated to increased DNA synthesis by the tumor cells than by the nonmalignant cells, and the frequency varied among the 8 tumor groups studied; a factor in serum blocks the stimulating ability; fresh tumor biopsy cells that did not stimulate could be made stimulatory after antibody was eluted from the cells by washing with glycerine; cells from local lymph nodes draining the tumor were not stimulated in 6 of 8 patients even though the same tumor cells stimulated peripheral circulating lymphocytes; interrelationships of host and tumor factors influence tumor-specific immune response qualitatively and quantitatively, making interpretations difficult until additional quantitative information accumulates and the conceptual schemes in immunobiology become more mature.

4. In studies (with BALB/c spleen cells immunized against the EL 4 lymphoid tumor) on host regulation of function and development of cytotoxic lymphoid cells (measured by a rapid  $^{51}\text{Cr}$  release), it was found that cytotoxic cells, once developed, are limited by their lifespan and inaccessibility to target cells (including antibody coating) and function essentially independently of other host regulation; by contrast, production of "killer cell" lymphocytes is highly sensitive to host regulation; a "killer cell" can destroy more than one target cell during its lifetime.

5. In assays of serum-mediated cytotoxic and cell-mediated immunity, involving electronic scoring by image analysis with several types of human tumor cells in cul-

ture, some specificity of cancer types and in autochthonous systems was observed, but reactivity among different cancer types makes interpretation difficult; in cell-mediated assays, the reactivity did not follow the HL-A antigens on different target cells.

6. Three assays were studied in acute lymphocytic (ALL) and acute myelogenous leukemia (AML) patients: delayed hypersensitivity to tumor cell extracts, mixed lymphocyte culture, and lymphocyte cytotoxicity; the assay results obtained with patients at about the same time did not correlate well among the 3 test systems used; however, skin tests with autologous membrane extracts of blast cells correlated with disease state (positive in most patients tested and in only 30 % of patients in relapse), and skin reactivity became negative shortly before or at the time of clinical relapse; cross-reactions between ALL and AML did not occur, and no reactions were detected with the patient's own normal cells; a rebound phenomenon (increase with lymphocyte stimulation) occurred about 10 days after cessation of chemotherapy.

In Dr. Bloom's summary of this session, he referred to key questions raised by Dr. Herberman:

1. Are different tests measuring the same or different antigens? How specific are they?
2. Or, are they measuring different phases of an immune response?
3. What is the relationship of each test to various clinical states and to resistance against tumors?
4. Which tests (if any) will be useful in monitoring immunotherapy?

He added additional questions raised by the presentations of the session:

1. What are the roles of T-cells, which are involved in graft rejection, and of B-cells?
2. Tumor cells can be killed *in vitro*, but can antibodies be made that block *in vivo*? Are the same kinds of cells involved?
3. Does blocking provide an escape from tumor immunity and protection from killer cells?
4. Why are reactions of lymphocytes from nodes different from those in peripheral blood?
5. Can B-cells kill without complement?

Considerable discussion was held on blocking antibodies. Timing in relation to tumor growth is important: At 3–7 days, sensitized lymphocytes appear; blocking factors appear at 2–5 days. Blocking needs to be defined at the actual target cell site or at the lymphocyte-forming or activation site. Elution of blocking factors from cell surfaces gives 2 factors: one with a mol wt >100,000 (an immunoglobulin) and one with a mol wt <10,000; when they are from different tumors, they do not cross-react; neither alone will block, but will if used together. It appears that there are frequently more circulating cancer cells than ever become tumors; does blocking play a role? Even under the best conditions yet developed, full blocking cannot be attained and well-established tumors continue to grow. An interesting suggestion was made: Why not absorb serum with columns containing lymphocytes? Two investigators stated that they would try such a procedure.

Dr. Black again reminded everyone of the importance of the patient with his caution: "We are hearing about elegant techniques applied to abstract issues" and

with his apothegm: "Around every tumor is a patient." His "Around every lymphoreticuloendothelial system is a tumor" is more difficult to classify.

## SESSION 6

Session 6 was on heterogenization. Dr. Paul introduced the session by citing a number of problems with the postulate that syngeneic skin-graft rejection results from a virus-induced antigenic alteration of normal tissues. Rejection may not be due to cell-mediated immunity (*see* Dr. Mariani's studies). If virus-induced changes are extensive in the case of nontransformed cells, immunotherapy may have less chance of success, since the number of targets might be enormous. Dr. Paul next discussed antigen recognition: Four or more steps are involved and three cell types participate (macrophages or other cell types; T-cells; and B-cells). Two antigen-recognition levels exist with T-cells (which occupy a central position in immune-response initiation): 1) acceptance of antigen at the cell-surface receptor sites; and 2) interaction and modulation by gene products of immune-response genes. In the influence of T-cells on B-cells, which may secrete immunoglobulins, are the B-cells different forms of the same line, or are they separate lines? What is the precise limiting step for antibody production? What is the role of immune-response gene products? If they are absent, what are the mechanisms for activating cells? Dr. Paul concluded that it is premature to be certain that successful immunotherapy or immunoprophylaxis can be achieved, but tools are in hand to move toward the goal.

Highlights of this session include the following:

1. Results from studies on partial rejection of isogenic skin grafts from leukemic BALB/c donors in recipients preimmunized with virus-induced tumor cells suggested that incomplete rejection resulted from decreased host reactivity, possibly as a consequence of virus infection; indirect evidence suggested that leukemic cells within the graft may cause graft rejection (more direct evidence was presented by Dr. Mariani).

2. Two experiments were performed to ascertain whether Concanavalin A (Con A) enhances the immunogenicity of EL 4 lymphoid leukemic cells (to produce anti-EL 4 cytotoxic lymphoid cell activity) by providing antigenic "carrier" determinants; the results suggested that the effect of Con A may be directly on the immune system and not on the tumor-associated surface antigens.

3. With MCA-induced and simian virus 40 (SV40)-induced tumors of mice, influenza virus-infected tumor cells yield homogenates capable of inducing tumor immunity; homogenates from cells not infected do not induce this type of immunity; this technique may prove useful for isolating tumor-specific transplantation antigens from human tumors.

4. In studies with the Gross passage A virus system, the number of tumor cells present in the graft correlated with rejection of syngeneic skin grafts (with irradiated skin grafts and with tumor cells injected into skin grafts); total-body irradiation and neonatal thymectomy had no effect on rejection rates; direct injection of virus as cell-free filtrates of leukemic tissues and irradiated ascitic fluid into newly placed grafts led to no rejections, even though disseminated leukemia later resulted.



In closing the session, Dr. Mariani cited the need for clearer criteria for describing phenomena, since different systems, definitions, and criteria are being used resulting in some confusion. Two comments of note from the discussion were: 1) Dr. Svet-Moldavsky, who introduced the concept of heterogenization, also used nononcogenic viruses, and rejections still occurred; and 2) immunologic enhancement should also be studied in *weakly* antigenic tumors and not only in strongly antigenic ones such as SV40 virus-induced tumors.

## SESSION 7

Session 7, the final one, was on diagnosis and immunotherapy of cancer. Dr. Klein opened the session by expressing pleasure that new programs with funding had recently been established in cancer immunology at the national level. He expressed concern that many interested in cancer chemotherapy and in other forms of cancer treatment had been reluctant to support work in immunotherapy. However, more recently, immunotherapy work is receiving new support. An excellent beginning has been made with the first model devised by Dr. Rapp, but it is really only the "Model T" and much progress can be expected.

Highlights of this session 7 include the following:

1. The effect of BCG infection on syngeneic transplantable tumors was studied in guinea pigs and mice (BCG and tumor cells mixed *in vitro* prior to intradermal injection, or BCG injected into intradermal tumor nodules); tumor cell killing occurred when BCG at a restricted-sized tumor site produced a chronic inflammatory reaction of the delayed-hypersensitivity type; presensitization with BCG had no therapeutic effect; a preparation of BCG cell walls in oil droplets also killed tumor cells; the activated histiocyte was suspected as being responsible for the cell killing; in the guinea pig, granulomatous reactions with intense proliferation of stromal reticuloendothelial components and histiocytes occurred at both the tumor sites and in regional lymph nodes containing metastatic tumor cells; regression was complete and metastases were eliminated.

2. Immunization with Gross virus inhibited growth of spontaneous AKR leukemia; sequential therapy with drugs followed by immunotherapy could eradicate the leukemia (immunotherapy eradicates only if the tumor cell number is  $\leq 10^5$ ); the evidence indicates that immune tolerance was absent.

3. In clinical trials with acute lymphocytic leukemia patients, active immunotherapy (BCG + neuriminidase) produced longer remission periods, and treatment with vincristine or adamantadine abolished the effect of immunotherapy; changes in protocols as the trials progressed make interpretation of results somewhat difficult; microlymphoblastic and prolymphoblastic forms of cells are sensitive to immunotherapy and to chemotherapy, and macrolymphoblastic forms are sensitive to chemotherapy.

4. Correlations exist between a patient's immune response to his tumor (melanomas and skeletal and soft tissue sarcomas) and the extent and progression of his malignant disease, and attempts were made to enhance the immune response by active immunotherapy with autologous or homologous tumor cells plus BCG; an increase in antibody titer occurred in patients immunologically competent; this rise

was sometimes associated with tumor regression. (The studies are too recent to permit follow-up for survival data.)

5. Review of data from patients challenged with intradermal dinitrochlorobenzene or purified protein derivative of tuberculin (delayed hypersensitivity) indicated that resolution of more than 95 % of premalignant keratoses, superficial basal cell carcinomas, and squamous cell carcinomas occurred in 120 patients, with no recurrences for up to 8 years; the higher level of responsiveness in such patients permits early diagnosis, and lack of reaction in normal skin allows healing to proceed; immunity could be transferred to nonsensitized patients by means of peripheral leukocytes from sensitized patients; regressions of metastatic carcinomas have been produced in patients followed from  $1\frac{1}{2}$ – $3\frac{1}{2}$  years in 10 of 18 cases (immunologic competence must be present if regression is to occur).

6. In a large series of patients with various kinds of cancer, a small number were found to have serum antibodies to cancer tissue before immunization (with cell-free tumor preparations) and, depending upon the assay used, 30–60 % had detectable antibody after immunization; cancer specificity was questionable, and no clear relationship with clinical response was noted.

7. Alpha-1-fetoprotein, though not a “tumor antigen,” is useful in diagnosing and following the clinical course of patients with hepatomas and certain embryonal teratoblastomas; reports indicate positive tests in 50–95 % of hepatoma patients, one-third of patients with embryonal testicular tumors, <10 % of patients with tumors metastatic to the liver, nearly 100 % in infants under 8 weeks of age, <1 % in patients with viral hepatitis and other inflammatory conditions, and negative in subjects with regenerating liver.

8. CEA can be found in noncancerous lesions of colonic mucosae (polyps, ulcerative colitis); the localization of CEA as visualized with fluorescence techniques is not always on cell membranes; specificity is also complicated by the finding of a CEA cross-reacting protein in colonic mucosa, lung, spleen, and plasma.

9. A new triple  $\gamma$ -emitting isotope, double-antibody radioimmune assay for CEA was described, sensitive in ng quantities and coupled to a computer program to facilitate calculation of results; it is valuable in following isolation and characterization of CEA.

10. CEA isolated from several digestive tract tumors was obtained in 6.8S (more commonly) and 10.1S molecular sizes, with considerable variation in yield from different tumors; amino acid analyses of CEA from different tumors were similar; amino acid-sequencing determinations are under way; CEA-like material is being produced in tissue cultures of malignant cells.

11. The status was presented of a clinical investigation on CEA involving 45 institutions with subjects on 2 protocols; the highest positive titers (about 85 %) was found in patients with carcinomas of the colon, stomach, or lung, and >90 % of healthy volunteers showed negative titers; the test will aid the clinician in monitoring the course of patients with known carcinoma, but it is doubtful that it will be useful as an effective screening device.

Discussion within this session brought forth a number of points. While BCG appeared to produce tumor regression only when injected locally intradermally,

MER produced effects even when given at a site distant from the tumor. It was further emphasized that patients must be immunocompetent for therapeutic effects to occur, and, if not, they perhaps should not be included in series results aimed at evaluating possible immunotherapy possibilities. Some of the methods used for preparing tumor materials for cancer immunology studies could be expected to destroy many antigens. Several participants reported that preliminary immunotherapy trials were under way.

### CONCLUDING REMARKS

Although some comments suggested concern that some of those in cancer chemotherapy work had perhaps held up development of cancer immunotherapy, it should be noted that better designed clinical trials in immunotherapy are needed, and we can learn much from those in cancer drug therapy who pioneered and developed soundly designed clinical trials (20, 21), usually having available only the far advanced patient already unsuccessfully treated by other means. Cooperation among clinical investigators specializing in particular modalities of therapy is essential. Our lack of full success in cancer prevention and cure demands it. One only needs to look at the statistics, the slides we see at meetings, and the patients themselves to feel the obligation to help each other as we strive to improve the lot of the cancer patient.

The work reported at this Conference illustrates the dynamic state of cancer immunology. Although some confusion exists on several issues, the leads now available indicate a great deal of movement toward realization of the potentially great benefits to be derived from immunology. Although considerable work will be required to quantify and clarify much in the field, I am optimistic that rapid progress will be made in the next couple of years. We did make the decision this past year at the National Cancer Institute to create a special program in immunology and earmark additional funds for its initiation. Dr. Terry, who heads the program, will discuss the plans for this new enlarged effort.

I would like to close with some comments on the National Cancer Institute budget. Hearings before the Senate Appropriations Committee were held just a few days ago, and, as Director, I defended the \$430 million request in the President's Budget for fiscal year 1973 (22). This was the amount decided after many factors were considered, including projections of Federal Government income for the next year, the national debt, the state of the economy, competition among the many areas funded by the Government, etc. Prior to passage of the new National Cancer Act of 1971 (23) I had submitted a request for \$550 million. The Office of Management Budget decided \$430 million. When the new cancer act passed, it contained an appropriation ceiling totaling \$530 million, including \$30 million for new authorizations for added cancer control activities. A request for \$15 million for a cancer control program was then made to be included in the budget request; this was not allowed. To provide for a start on the new cancer control program, therefore, the budget allocations in the \$430 million request were revised to include \$4 million for this program.

At the Congressional Hearings in response to questioning, I indicated what could be done with an added \$100 million (22) should the appropriation reach the ceiling. I believe there are sufficient leads, momentum, and capability in the Nation to spend such amounts effectively, and we have program plans already developed for a program larger than this size (24, 25). I doubt if the appropriation will reach the \$530 million ceiling, in part because of deficits in government monies; my best estimate is between \$480 million and \$500 million.



We are in exciting times in cancer research. The spirit is up. The Public is with us. The funding has increased from \$181 million only 3 years ago. There is one danger, however, that has concerned the scientific community: The Public may expect all cancers cured or eliminated in the next 2 or 3 years. Great care was taken by many in testimony and in contacts with the press to point out the unlikelihood of such a quick and easy success. But over the next decade, great progress can be expected (26), and some of the things we have had presented at this Conference are the beginnings of a whole new area which will have excellent payoff. We have many leads, new plans, new funds, and plenty of work to do. To all working in cancer immunology, I wish you great success.

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## **Perspectives on the Conference on Immunology of Carcinogenesis<sup>1</sup>**

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It is always difficult, and usually not profitable, to speculate about the future of anything, and it is clearly impossible to say much that will be useful about so complex an issue as the future of tumor immunology. The only likely virtue to the remarks I will make will therefore be their brevity.

One cannot discuss the future without considering the past. As you know, the history of tumor immunology has been quite a checkered one. I will not detail the various progressor and regressor states that it has gone through, nor will I try to deal with the various factors that have perhaps impeded its progress.

Clearly, important work has been performed in the past, and the literature on tumor immunology is extensive. At the present time, many excellent immunologists are developing an interest in tumor immunology, and one of the immediate tasks in this field is to devise mechanisms that will enable our colleagues to review comprehensively the literature on tumor immunology. This will be important so that time and precious resources are not devoted in the next few years to repeating experiments that have long since been performed. An appreciation of the past will be quite important in helping us to establish our future directions.

Dr. Carl Baker beautifully summarized that part of the present state of tumor immunology that was touched upon at this Conference. There is obviously more than has been considered here, but this is not the occasion for a detailed analysis of the state of tumor immunology.

What is clear, and what Dr. Baker mentioned in the last few minutes of his presentation, is that the progress made in the past has generated great enthusiasm in the present, and that the effect of this enthusiasm is that more money has been made available for research on tumor immunology and, perhaps more importantly, every indication is that still more money will be made available for support of research in this area.

This provides real opportunities, but it also provides great dangers. Expectations are being generated which, if not fulfilled, will again send support for tumor immunology into an unjustified and premature decline. To guard against this, we must very carefully consider where we are going; in other words, we really must consider our future. There is a subtle quote from Galsworthy which says, "If you do not think about the future, you cannot have one."

Last fall, in a very unsubtle way, Dr. Baker, speaking to some of us at the National Cancer Institute, put it much more bluntly. He said: "Listen, fellows, if you really think that tumor immunology is ready for a major expansion, you had better show me some plans." In other words, if we want resources for the support of this area, we need to do some planning for the future.

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<sup>2</sup> National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

As a consequence of this challenge, Dr. Herberman, Dr. Rapp, and I spent a great deal of time formulating an immunology program plan. When this plan has been finalized, we hope it will provide the nucleus for a national strategy in the development and application of immunology to the problems of malignant disease in man.

Again, because of insufficient time, I am not going to detail the plan as it currently stands, but when a final draft of this plan has been formulated in the near future, copies of the plan will be made available to those who are interested.

Basically the plan is designed to achieve a number of goals. First, we are endeavoring to pinpoint those areas of investigation in immunobiology that are relevant to tumor immunology and that require further exploration.

Second, we are attempting to consider how immunology relates to the etiology, prevention, diagnosis, and therapy of malignant diseases and to pinpoint the areas in each of these categories where perhaps insufficient relevant work is being done and where new or different work might be stimulated.

Third, we hope to plan ahead for resources that either are or will be required in order to facilitate progress at the maximal, possible rate in the areas of etiology, prevention, diagnosis, and therapy.

When these needs are identified, priorities will be established, and work in these areas will be supported by a variety of financial mechanisms, with the emphasis being on contracts.

In terms of this type of advanced planning, let me pick out one minor example. We have had some discussion at this Conference concerning carcinoembryonic antigen and the possibility of its use as a diagnostic cancer test. In evaluating this test, as well as many other tests proposed in the past, we must have available sera from patients with known neoplastic diseases and non-neoplastic diseases, and sera from "normals." For these sera to be useful, they have to be well validated and have a good distribution as regards age, sex, and race. There has always been a significant lag in evaluation of a test because the necessary panel was not available and it has taken some time to put such a panel together.

Having recognized this problem, it seems reasonable to develop a resource that will improve the speed of evaluation of supposed diagnostic tests. It is certainly possible to establish a bank of appropriate sera, to store the clinical information in computers and to make this bank available to help in the rapid evaluation of cancer diagnostic tests. This project has indeed been initiated; in the near future, a bank of sera will be available for the evaluation of cancer diagnostic tests. One consequence of this type of planning and the expenditure of contract dollars to implement the planning should be to shorten the time between the description of a possible cancer diagnostic test and its delivery to the public.

In my comments, I have used a number of words which generally are scare words as far as some portions of the academic community are concerned. Such words as "planning," "relevance," "priority," and "contracts" frequently raise the hackles of the academic community; yet these are words and concepts with which we really must deal. It will be necessary to get rid of the emotionalism associated with many of these words in order for us to utilize the resources being made available in a maximally effective manner. By drawing on the collective wisdom of the national and indeed the international community, we hope that whatever planning we do will be intelligent, that the definitions of relevance and priority will be appropriate, and that, as a result, we will be able to achieve our goals more rapidly.

A few words are in order concerning how we will go into the future; *i.e.*, what the quality of the future is likely to be. It is trite to say that a close relationship



between clinicians and bench scientists will be required. We all say it and then frequently ignore it. The point has been made repeatedly at this Conference, and I think it is worth underscoring that the patient tells us what is relevant and important and that the sharp-eyed and sharp-minded clinician translates what the patient is telling us and passes it along to the bench scientist, who in turn must retranslate this information into an experimental protocol.

If the road from patient to clinician to scientist is too long or if the scientist ignores the clinician, then progress will certainly be slower than it ought to be. Reciprocally, if the clinician ignores the scientist, he is likely to establish improper and time-wasting clinical trials that will also impede progress.

Immunotherapy, discussed at this Conference, is certainly an area in which there are crucial interrelationships between the scientist at the bench and the clinician. We have heard a great deal that is quite exciting and very important, and it is clear that work in immunotherapy will be moved ahead as rapidly as possible.

We cannot ignore, however, the history of medicine, in which numbers of therapeutic approaches have appeared to be exceedingly important and very exciting, and yet have not survived for very long. One can think, for example, of the utilization of gastric freezing in gastrointestinal bleedings. Perhaps more relevant was the assumption that steroids, when they first came upon the scene, were the cure-all for everything. Now steroids, clearly, are a very useful part of the physician's armamentarium, but it has taken some time and some study to define circumstances in which they can be used successfully and also to determine those circumstances in which they are useless or even harmful.

We must realize that our current approaches to immunotherapy are really quite primitive. Dr. Jan Stjernswärd made a comment to me the other evening which I think is really quite appropriate: We should recognize that we are perhaps several thousand years behind the African medicine men, who over that period of time have been scarifying their patients, and instead of putting bacille Calmette-Guérin in the scarified area, they have been rubbing in cow dung. It is not unlikely—and I say this not lightly—that the effects may be quite similar.

If we are to have real progress in immunotherapy, as well as in the rest of tumor immunology, we shall have to take the time to look at mechanisms, as well as to move empirically toward doing what seems to work. Unless we do this, our capacity to generalize and to develop principles of therapy based on fundamental immune mechanisms will be severely impaired. A close working relationship between the clinician and the laboratory scientist will be a key element in our ability to make significant advances. We are also going to require a submergence of professional egos that has probably not occurred previously in this part of biomedical research. The very complexity of the task ahead will require team efforts and interlaboratory and interinstitution cooperation and collaboration. There will have to be more emphasis on the meaning of what is published and less emphasis on who published it first. There will also have to be greater concern for how we can apply what is being published as rapidly as possible to the ultimate benefit of the cancer patient. Certainly the fact that we all share this common goal will make it easier for us to develop an appropriate spirit of joint effort as we proceed.

A comment about the basic science of immunology is also in order. Some basic scientists fear that the emphasis on planning and application I have mentioned in this presentation will eliminate support for basic research. Let me strongly indicate that precisely the reverse is true. All the planning and the attempted applications can only serve to underscore the need for continued and expanded fundamental research in immunology. Obviously, if we had all the answers, we could be out sitting

around the pool enjoying the sun right now, and we would not have to be in here worrying about where we are going. Continued development of an understanding of the fundamental nature of the immune system is clearly the key to our attempts to apply the system to the prevention, diagnosis, and cure of cancer.

In summary, then, and in a way to paraphrase what Dr. Baker said, I think the future for tumor immunology is quite bright—perhaps brighter than it has ever been. Resources will be made available. It is up to us to expand the necessary energy and intelligence to convert these resources, and the potential of the future, into reality.

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